1994

Role of Curved DNA in Promoter Recognition With Bacillus Subtilis and Escherichia Coli RNA Polymerase.

Cheryl Anne Nickerson
Louisiana State University and Agricultural & Mechanical College

Order Number 9502134

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Nickerson, Cheryl Anne, Ph.D.

The Louisiana State University and Agricultural and Mechanical Col., 1994

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300 N. Zeeb Rd.
Ann Arbor, MI 48106

ROLE OF CURVED DNA IN PROMOTER RECOGNITION WITH *Bacillus subtilis* AND *Escherichia coli* RNA POLYMERASE

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirement for the degree of
Doctor of Philosophy

in

The Department of Microbiology

by
Cheryl Anne Nickerson
B.S., Tulane University, 1983
M.S., University of Missouri, Columbia, 1988
May,1994

DEDICATION

This work is dedicated to my parents, Dr. and Mrs. Max A. Nickerson. It has only been through their constant love, support, encouragement, and unwaivering belief in my abilities, that I have found the strength necessary to meet any challenges that have faced me. I can state without hesitation that had it not been for their patience and understanding, this degree could never have been realized.

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to my major professor, Dr. Eric C. Achberger, for providing me not only with the laboratory facilities necessary to conduct my research but also the opportunity to intellectually challenge myself as a scientist. His advice as a mentor and colleague is highly valued and will serve me well in the future.

Sincere gratitude is also extended to Dr. John Battista for his encouragement and invaluable advice and insight into my scientific pursuits both here at L.S.U. and in future endeavors. He was always very supportive and generous with his time.

Special thanks to Dr. H.D. Braymer, who was always very helpful and encouraging, and genuinely interested in my future as a scientist.

My thanks also to Dr. Randall Gayda and Dr. Ding Shih for their advice, patience, and constructive criticism of this dissertation. Their generous donation of time in this effort was greatly appreciated.

I would also like to extend my deepest appreciation and gratitude to my fellow graduate students in the microbiology department for their friendship and encouragement. They helped make my stay at L.S.U. an enjoyable one. Special thanks to my dear friend, Anand Immaneni, whose companionship, generosity, and loyalty will never be forgotten. My fellow lab colleague, Doug Stemke, was always very patient and helpful, his kindness was most appreciated. My thanks also to Evie Vandergrifft, for her valued friendship and unique outlook on the "graduate

experience", to Tim Beary, for his helpful advice and encouragement, and Mark Ott, for his patience, support, and friendship.

My heartfelt thanks are also extended to Saurabh Singh, Karuna Murray, Marilyn Morse, Mena Cho, Kevin Riley, Michael Raggio, Dr. C.N. Riley, and Dr. C.A. Buddy, for their assistance, patience and support. Their friendship and unselfish donation of time made my job much easier. I would also like to extend my deepest appreciation to my wonderful friend, Gopal Hara. His support and encouragement were most appreciated, and his courage admired.

A special acknowledgement to my grandparents, Mr. and Mrs. Raymond Bartee, Mr. and Mrs. I.J. Nickerson and to my uncle Mr. Herald Bartee. As with all endeavors in my life, they have always supported, encouraged and inspired me to do my best.

Finally, and most important of all, I wish to thank my parents for their constant love, support and encouragement throughout my life. Had it not been for them, I could never have found the strength, determination, and fortitude necessary to pursue this degree.

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ABSTRACT

Intrinsically curved DNA upstream of the RNA polymerase binding site, the promoter, influences transcription in *Bacillus subtilis*. In this study, curved DNA upstream of the -35 region was examined for the ability to influence transcription in *Escherichia coli* and *B. subtilis* through the use of hybrid promoters. These promoters were constructed by substituting the curved DNA from two *B. subtilis* promoters, Bal129 and Alu156, for the DNA upstream of the -35 region in the lambda phage promoters p_R and p_L. The hybrid promoters were designated Balp_R, Alup_R, Balp_L, and Alup_L. Using transcriptional fusions between each promoter and the chloramphenicol acetyltransferase (*cat*) gene, CAT expression was measured in *B. subtilis* and *E. coli*. The addition of curved DNA significantly increased transcription from the Balp_L and Alup_L promoters compared with λp_L in *B. subtilis*. In *E. coli*, transcription was not significantly affected by the presence of the curved DNA. In the competition binding assay, *E. coli* RNA polymerase bound DNA fragments containing the Balp_R or Alup_R promoters much more effectively than those with the original λp_R. However, the *E. coli* enzyme did not discriminate among the promoters in the p_L series. In general, *B. subtilis* RNA polymerase displayed greater affinity for the hybrid promoters than it did for the original λ promoters. DNA supercoiling affected the binding of *E. coli* RNA polymerase to the hybrid promoters when the interaction was analyzed in a gel retardation assay. On a supercoiled DNA template, the hybrid promoters were bound by RNA polymerase more effectively than was the original λ promoters. DNaseI footprinting was used to

characterize the interaction between *E. coli* RNA polymerase and the hybrid promoters. This assay provided evidence that the *E. coli* RNA polymerase tightly wraps the curved DNA when binding the Balp_R and Alup_R promoters. No evidence of DNA wrapping was observed with the original λp_R. We propose that the tight wrapping of the upstream curved DNA around the RNA polymerase may facilitate and/or be required for the subsequent steps of DNA untwisting and strand separation.

INTRODUCTION

A critical parameter in studying the regulation of a gene is to understand the activity of its promoter, which is the site of transcription initiation. The initiation of transcription is a vital step in the control of gene expression. The regulation of gene expression at this stage is economical for the cell in that a particular RNA transcript is not synthesized in any appreciable quantity until it is required by the cell.

The basic components involved in controlling transcription initiation are: 1) RNA polymerase, the DNA-dependent RNA polymerase which catalyzes RNA synthesis, 2) promoters, the unique sequences of DNA that define the start of a gene, bind RNA polymerase and initiate transcription, 3) regulatory proteins, proteins which function to either activate or repress initiation of transcription via interaction with the RNA polymerase and/or the DNA.

RNA polymerase. Since the RNA polymerase is the only enzyme essential to transcription, it is logical that any discussion of control of gene expression begin here. In prokaryotes, a single type of core RNA polymerase is responsible for the synthesis of all mRNA, tRNA, and rRNA molecules. Bacterial RNA polymerases are large, multimeric enzymes of impressive complexity. The *E. coli* RNA polymerase holoenzyme has a molecular weight of approximately 480,000 daltons, and a subunit composition of α (alpha, 36,512 daltons, present as a dimer), β (beta, 150,618 daltons), β', (beta prime, 155,613 daltons), ω, (omega, 10,105 daltons) (Burgess, 1976), and σ,

(sigma, 70,263 daltons for σ^{70}, the major vegetative σ (McClure, 1985). In *E. coli*, there are about 3,000 molecules of RNA polymerase holoenzyme (Burgess, R.R., 1976) of which, approximately half are actively engaged in transcription (Ingraham, *et al.*, 1983).

The complexity of the RNA polymerase enzyme is a reflection of its funtional diversity. RNA polymerase recognizes the transcriptional start site on the DNA, (the promoter), untwists and separates the strands of the DNA helix, incorporates complementary ribonucleotides without a primer, elongates the transcript, and recognizes specific transcription terminators. In addition, RNA polymerase interacts with a wide variety of protein factors that modify its enzymatic activities. The holoenzyme form of RNA polymerase consists of two basic components, the core enzyme, ($\alpha_2\beta\beta'\omega$), and the σ subunit. Only the holoenzyme form is capable of initiating transcription. The core enzyme is catalytically active once initiated, but it binds DNA in a nonspecific fashion in solution (Hansen and McClure, 1980). The σ subunit confers on the holoenzyme the ability to recognize and bind promoter sequences. There is approximately a 10^4-fold increase in promoter specificity upon the association of sigma with the core enzyme. Shortly after the initiation of transcription, the sigma factor is released from the holoenzyme, leaving the core enzyme to complete transcriptional elongation and termination (Hansen and McClure, 1980). The released sigma factor may associate with free core RNA polymerase and the process of transcriptional initiation can begin again. The technique of multiple sequence alignment (Gribskov and Burgess, 1976; Helmann and Chamberlin, 1988; Stragier and Bouvier,

1985) as well as genetic suppression experiments (Zuber *et al.*, 1989) have identified four general regions which are highly conserved among the primary sigma factors in many bacteria. Two of these conserved regions are very basic and have a postulated role in DNA binding to the -10 and -35 regions of the promoter. In addition, these two regions may be involved in DNA strand melting. The remaining two conserved regions are acidic, and exhibit lower conservation of sequence than the two basic consensus sequences. These acidic consensus elements may be involved in core binding to the α subunit.

Under normal growth conditions, the major σ found in the *E. coli* RNA polymerase holoenzyme is σ^{70}, the product of the *rpoD* gene. However, *E. coli* possesses several other sigma subunits in addition to σ^{70}. These different sigma factors enable the core enzyme to recognize and utilize distinct promoters that have sequences unique from those recognized by the primary σ factor. The substitution of one σ for another allows the cell to modify the RNA polymerase to recognize a new class of genes (Burgess and Travers, 1970). Alternative sigma factors in *E. coli* are activated by adverse conditions like heat shock or nitrogen starvation. The *E. coli rpoH* gene encodes a 32,000 dalton protein, σ^{32}, which directs RNA polymerase to initiate transcription from promoters of heat shock genes (Neidhart, *et al.*, 1984). Yet another σ subunit, σ^{54}, regulates gene expression during conditions such as nitrogen starvation (Garcia, *et al.*, 1977).

In contrast to *E. coli*, *B. subtilis* is a sporulating bacteria, and therefore undergoes a complex developmental process. It is not surprising then, that in *B.*

subtilis, many sigmas are dedicated to the sporulation process. As in *E. coli*, these sigma factors direct transcription from specific genes. Many of these alternative sigmas are involved in regulating the temporal patterns of gene expression during sporulation. The RNA polymerase core enzyme of *B. subtilis* is remarkably similar in structure to that of *E. coli* (Gitt, *et al.*, 1985). The *B. subtilis* core RNA polymerase contains the additional subunits ω and δ (Pero, *et al.*, 1975). The δ subunit is involved in promoter discrimination, and allows the holoenzyme to differentiate between strong and weak promoters (Achberger and Whitely, 1981; Whitely *et al.*, 1982). In *B. subtilis*, σ^A is found associated with most of the holoenzyme purified from vegetatively grown cells, and the DNA sequence has a striking homology with its *E. coli* counterpart, σ70. Indeed, RNA polymerase from *B. subtilis* containing σ^A recognizes promoters with the same consensus sequences at the -10 and -35 regions as does σ^{70} in *E. coli* (Henkin and Sonenshein, 1987; Kenney and Moran, 1991; Moran *et al.*, 1982).

In addition to σ^A, *B. subtilis* has several other minor sigmas that are also found in vegetatively grown cells, including σ^D, σ^H, and σ^L. The σ^D containing RNA polymerase transcribes the genes involved in flagellar development and chemotaxis. A function of σ^L in *B. subtilis* is to regulate expression of the levanase operon (Debarbouille, *et al.*, 1991). Sigma H is necessary for the onset of sporulation and is active in the mother cell. However, the concentration of σ^H dramatically increases during the initiation of sporulation. Indeed, σ^H holoenzyme transcribes many sporulation-specific genes during the early stages of sporulation.

Alternative sigma factors in *B. subtilis* play their major role in differential gene expression during sporulation, a condition which represents a developmental process with two distinct cell types. In *B. subtilis*, a trigger for morphogenetic change from vegetative growth to production of endospores is starvation. Under the conditions of nutrient depletion, sporulation-specific genes are transcribed and vegetative protein production ceases. Several sporulation-specific sigma factors appear sequentially during this process to activate transcription from specific promoters. Sigma factors σ^E (Jones and Moran, 1991), σ^F (Margolis *et al.*, 1991), σ^G (Coppolecchia *et al.*, 1991; Karmazyn-Campelli *et al.*, 1989; Rather *et al.*, 1990), and σ^K, (Stragier *et al.*, 1989), have been identified as sporulation-specific by genetic analysis. Results from these studies have shown that each of these sigma factors is essential for unique gene expression in distinct stages of sporulation. Both σ^A and σ^H are active in the earliest stages of sporulation. Sigma H is required for the onset of sporulation. Mutants which inactivate the structural gene encoding σ^H, *spoOH*, prevent the initiation of sporulation (Dudnau *et al.*, 1989; Wu *et al.*, 1991). In addition, σ^E, which appears to be active only in the mother cell, is also required in the initial stages of sporulation. Sigma F, although produced in the mother cell prior to asymmetric cell division, is not active until after septation. In addition, σ^F is required for expression of σ^G, which is responsible for transcription of forespore specific genes. The activity of σ^K is restricted exclusively to the mother cell, and is necessary for production of the *cot* genes, which are the structural genes encoding the endospore coat proteins (Stragier,*et al.*, 1989).

It should be noted that numerous operons of *Bacillus* are associated with overlapping or tandem promoters, each recognized by a different sigma factor (Johnson, *et al.*, 1983). Such complex promoter arrangements may allow for greater flexibility in response to changes in nutritional conditions or developmental stages of the bacterium.

Alternative sigma factors were initially discovered in phage-infected cells of *B. subtilis*. Those sigma factors encoded by the *B. subtilis* lytic phages SP01 and SP82, were involved in controlling the transition of expression from middle to late genes. The early genes, expressed immediately after infection of the bacteriophage, were transcribed by the σ^A holoenzyme of *B. subtilis*, whereas phage-encoded sigmas were required to produce middle and late transcripts in a simple cascade of gene expression. The SP01-encoded gp28 has been well characterized and controls expression of middle genes, while gp33 and gp34 regulate late gene expression (Geidushik and Ito, 1982).

Promoters. Initiation of transcription is regulated by RNA polymerase-DNA interactions that occur at or near the promoter. Most prokaryotic promoters contain two regions important for RNA polymerase binding. These two regions are centered around DNA bases located at -10 and -35 with respect to the transcription initiation site. For the major form of *E. coli* RNA polymerase holoenzyme, the σ^{70} containing enzyme, there are two hexameric sequences conserved among these promoters, the -10 sequence (*i.e.*, 5'-TATAAT-3') and the -35 sequence (*ie.*, 5'-TTGACA-3'), which are separated by an optimal distance of 17 base pairs (Hawley and McClure, 1983). It has been

experimentally determined that the -35 region functions in initial binding of RNA polymerase, while the -10 region is involved in DNA melting. Presumably, the AT-rich base composition of the -10 region assists in the DNA strand separation during open complex formation. This is a logical assumption, since less energy is required to disrupt the weaker interstrand AT hydrogen bonds than those of GC basepairs.

The functional significance of these two consensus regions and the distance between them has been confirmed by genetic and biochemical studies. These studies involved measuring the *in vivo* affinity of RNA polymerase for wild-type and mutant promoters and and analyzing the polymerase-promoter contacts *in vitro*. Promoters with mutations in the -10 or -35 regions are profoundly affected in promoter strength. Promoter mutations which result in sequences that diverge from the optimal consensus sequence or spacing between the -10 and -35 regions lower promoter efficiency, while those which are more similar to the consensus sequence exhibit enhanced promoter activity. Presumably, the actual nucleotide sequence in the spacer region is not important, but rather, the distance between the two consensus regions may be the crucial factor. This distance may be a refection of a critical geometry necessary for a productive binding interaction between the polymerase and promoter.

Second site mutations in the σ subunit of *E.coli* RNA polymerase have been shown to compensate for the base pair substitutions in their respective promoters.In fact, genetic suppression studies have identified two regions of most σ factors which interact with the -10 and -35 region of promoters (Waldburger *et al.*, 1990; Siegle, *et al.*,1989; Kenney and Moran, 1991; Gardella and Susskind,1989).Moreover,substitution

of encoded amino acids in the conserved sequences of σ which interact with the -35 region have been shown to alter promoter specificity, such that holoenzyme containing the mutant σ directs transcription from promoters it would not normally recognize (Schmidt *et al.*, 1990).

The strength of an individual promoter sequence is defined in terms of the frequency of RNA initiation, and is related to the similarity with the consensus sequence and the DNA conformation. In reality, it is probably an interplay of the physical properties of the promoter, including flexibility, bending, base-pair stacking, DNA conformation, and neighboring nucleotide sequence, which "fine tunes" promoter function and modulates gene expression. In general, the assumption is made that a strong promoter will be utilized more efficiently and will generate a greater amount of gene product.

The use of chemical probes which result in DNA cleavage, such as dimethylsulfate (DMS) and the hydroxyl radical, in addition to the enzymatic probe DNase I, have provided detailed information on specific points of interaction between RNA polymerase and promoters. These studies have confirmed the importance of the -10, -35, and the spacer regions as sites where RNA polymerase interacts with the promoter. In particular, hydroxyl radical footprinting experiments have provided a very high resolution method for detecting specific contacts between protein and DNA. This high resolution is afforded by the size of the hydroxyl radical. It is the smallest chemical species which is used for footprinting and is extremely short-lived and highly

reactive. In addition, the hydroxyl radical shows no sequence specificity in its cleavage pattern, but is sensitive to distortion in the DNA helix, such as intrinsic bending.

Another method for detecting conformational changes in DNA is the technique of DNaseI protection mapping or footprinting. For this method, the protein of interest is allowed to bind to singly end-labeled DNA which contains the protein binding site(s).The protein-DNA complex is then exposed to digestion with DNase I. The basis of DNaseI footprinting is that DNA sequences which are bound by protein are protected against cleavage of the phosphodiester backbone of the DNA. Ideally, conditions are adjusted such that only one cleavage event occurs per DNA strand. Following DNase I-catalyzed nicking, the DNA fragments are separated by electrophoresis on a polyacrylamide gel designed for DNA sequencing. For every DNA bond susceptible to cleavage by DNase I, a band appears on the gel, which corresponds to the distance between the labeled end and the site of cleavage. In contrast, those regions of DNA protected from cleavage by bound protein will not generate a band on the gel, but rather, will appear as a gap within the ladder of DNA bands referred to as a footprint. Comparison of the pattern of bands generated from enzymatic cleavage to the DNA sequence reveal which nucleotides were involved in direct contact with the protein.

Regulatory molecules. There are some promoters which deviate significantly from the -35 consensus sequence, (*e.g.*, *araBAD*, $galP_1$, etc.). These promoters are fully active only in the presence of ancillary proteins, which aid RNA polymerase-promoter interactions. Some ancillary proteins are positive regulators, or

transcriptional activators, since transcription from dependent promoters can only occur in their presence. One of the most studied of these proteins, CAP (catabolite activator protein), controls the activity of a large number of genes important for bacterial growth and adaptation during glucose starvation (Crumbrugghe *et al.*, 1984). Site-specific DNA binding by CAP is stimulated by cAMP binding to CAP. *E. coli* preferentially uses glucose as an carbon source over other sugars. When glucose is available, the intracellular level of cAMP is low, and cAMP is unavailable to bind CAP. This phenomenon, known as catabolite repression, prevents expression of CAP-activated genes involved in the metabolism of sugars other than glucose, thus conserving cellular resources. Alternatively, as levels of glucose are depleted, the intracellular level of cAMP rises, the CAP protein forms an active complex with cAMP, and expression is activated at the promoters of catabolite sensitive operons. Upon binding near the promoters of relevant operons, the cAMP-CAP complex induces DNA bending (Fried and Cruthers, 1983; Kolb *et al.*, 1983; Liu-Johnson *et al.*, 1986; Wu and Cruthers, 1984, Zinkel and Cruthers, 1991). This protein induced DNA bending near the promoter may be important in formation of specific DNA conformations necessary to enhance transcriptional activity. At the *lac*P_1 promoter, it has been suggested that CAP exerts its affect on transcription initiation by increasing the rate of closed complex formation. Evidence from several laboratories indicates that CAP may activate transcription from CAP-dependent promoters by direct interaction with *E. coli* RNA polymerase (Blazy *et al.*, 1980; Mandecki and Caruthers, 1984; Pinkney and Hoggett; 1988 Straney et al., 1989). In this venue, it is interesting to note that *E. coli* RNA

polymerase also induces a bend in the helical axis of DNA upon binding at the promoter region (Kuhnke *et al.*, 1987; Huemann *et al.*, Bell, *et al.*, 1988). Perhaps this represents a necessary step in structuring the DNA in preparation for initiating synthesis of the transcript.

Bell, *et al.*, (Bell *et al.*, 1990) reported the effects of a number of mutations in the CAP protein which reduced the ability of the protein to activate transcription, but did not affect recognition or binding to target sites on the DNA. Several lines of investigation suggested that the α subunit of *E. coli* RNA polymerase played an important role in the control of transcription from promoters regulated by activator proteins like CRP, presumably CAP interacts directly with RNA polymerase at the C-terminus of the α subunit (Igarashi and Ishihama, 1991; Straney *et al.*, 1989; Bell *et al.*, 1990). Mutations in the C-terminal region of the α subunit have been found that prevent the activation of promoters dependent on CRP for their expression (Igarashi and Ishihama, 1991). Furthermore, deletions of as much as one-third of the α subunit C-terminus were shown to be dispensable for subunit assembly and core polymerase function, but the C-terminus was absolutely essential for activation by CRP-dependent promoters.

Just as ancillary proteins can affect transcriptional activity in a positive fashion, they can also repress gene expression. Negative regulation of transcription occurs when a repressor protein prevents RNA polymerase from initiating at the promoter. The repressor protein binds to a specific sequence of DNA called the operator, which usually overlaps the RNA polymerase binding site. When the operator sequence is

bound by the repressor, transcription is prevented. The lactose operon is a classical example of a metabolic system under negative transcriptional control (Jacob and Monod, 1961). The product of the *lac I* gene is an allosteric repressor protein, which contains two binding sites, one for the operator region, and one for the inducer molecule. The *lac* repressor protein may bind either substrate individually, but not both at the same time. When the *lac* repressor is bound to the operator regions, RNA polymerase is prevented from initiating transcription of the adjacent structural genes of the *lac* operon. However, mRNA synthesis is permitted when the inducer molecule is present, because the inducer binds to and alters the structure of the repressor, such that it can no longer bind to the operators. With the operators unoccupied, the promoter is available to bind RNA polymerse and initiate mRNA synthesis.

DNA looping is also involved in the negative regulation of transcription. Several operons (*i.e. gal* and *lac*) have dual operators in which repressor may bind and loop the DNA (Irani, *et al.*, 1983, Adhya, 1987, Mandal, *et al.*, 1990, Mossing and Record, 1986, Kramer *et al.*, 1987). In the *lac* operon, two operators are located upstream of the promoter. In the case of the *gal* operon, a repressor protein binds to two separate operators which are located on either side of the promoter. Binding of the *gal* repressor to the dual operator sites has been shown to result in the formation of a loop of DNA which contains the promoter. This looped DNA conformation blocks the RNA polymerase from binding to the promoter and initiating transcription.

DNA supercoiling. DNA supercoiling also plays a crucial role in the regulation of gene expression at the level of transcription. This is not surprising, since any parameter which can alter DNA topology might be predicted to affect transcriptional activity. In fact, several promoters have been shown to display dramatic sensitivity to changes in DNA supercoiling. Experiments with gyrase inhibitors have demonstrated that transcription from promoters associated with many genes (e.g., *rRNA*, *topA*, *gyrA*, *gyrB*, *ompC*, *ompF*) is affected by alterations in the level of DNA supercoiling (Dorman *et al.*, 1989, Graeme-Cook, *et al.*, 1989, Menzel and Gellert, 1983, Menzel and Gellert, 1987, Oostra *et al.*, 1981, Tse-Dinh and Beran, 1988). Frequency of transcriptional initiation has also been shown to vary as a function of the level of DNA supercoiling. In fact, the response of individual promoters to changes in supercoiling can be somewhat unique, since changes in DNA supercoiling which stimulate one promoter may decrease transcriptional activity from another. For example, genes that encode gyrase and topoisomerase I are associated with promoters which are sensitive to changes in supercoiling. An increase in negative supercoiling enhances expression from the *topA* (topoisomerase I) promoter and decrease expression of the *gyrA* and *gyrB* (gyrase) promoters (Menzel and Gellert, 1987; Tse-Dinh and Beran, 1988).

Circular DNA isolated from bacterial cells is a compact nucleoid structure which is negatively supercoiled. The level of DNA supercoiling in the cell is in a constant state of flux. Negatively supercoiled DNA is inherently underwound. It has been proposed that the DNA unwinding induced by negative supercoiling may activate transcription from certain promoters (*e.g.*, pBR322 promoters *rep*, *bla*, *tet*) (Brahms,

et al., 1985) by facilitating closed to open complex formation. The ability of negative supercoiling to facilitate open complex formation may be attributed to a localized decrease in base stacking at or near the promoter, which enhances the structural transition from closed to open complex formation.

Transcription initiation kinetics. Initiation of transcription is a multistep process which involves RNA polymerase binding to initially form a closed complex at the promoter, untwisting of the DNA, strand separation to form open complex, and finally, transcriptional initiation. Kinetic studies of the interaction of RNA polymerase holoenzyme with the promoter have been performed by a variety of techniques, including abortive initiation, run-off transcription, and nitrocellulose filter binding (Strauss, *et al.*,1981). The original model for transcription initiation was proposed by Chamberlin in 1974, (Chamberlin, 1974) and involved two kinetic intermediates:

$$R + P \leftrightarrows RP_c \leftrightarrows RP_o \leftrightarrows \text{RNA Synthesis}$$

In this model, the RNA polymerase (R) binds reversibly at the promoter (P) to form a closed complex (RP_c). The closed complex then undergoes a rate-limiting isomerization to open complex (RP_o) (Walter, *et al.*, 1967, Chamberlin 1974). In open complex, the DNA strands have separated for approximately 12 base pairs near the transcription start site. Once the open promoter complex has formed, transcription is initiated in the presence of the appropriate nucleoside triphosphates. After adding

between 2-9 nucleotides, the holoenzyme either releases the short oligomers and reinitiates transcription from the open complex in a process called abortive initiation, or releases sigma, leaving the core enzyme to carry out full length transcription. The conversion from repetitive abortive initiation cycles to a successful elongation mode has been attributed to the loss of the sigma subunit. When sigma is released the core enzyme clears the promoter and engages in full length transcription elongation (Hansen and McClure, 1980).

Evidence for additional kinetic intermediates involved in transcription initiation have emerged from binding and initiation studies carried out at different temperatures. These studies showed the existence of at least one new RNA polymerase-promoter complex in between closed and open complexes. When studied under different temperatures and ionic strengths, the isomerization of the inactive intermediate RP_c to another intermediate, RP_i, was the rate-limiting step. RP_i represents a complex which has undergone partial unwinding of its DNA strands (Buc and McClure, 1985). The additional intermediate can be included in schematic form as follows:

$$R + P \leftrightharpoons RP_c \leftrightharpoons RP_i \leftrightharpoons RP_o \leftrightharpoons \text{RNA Synthesis}$$

The closed complex (RP_c) and the "new" partially unwound species (RP_i), represent conformationally distinct forms of RNA polymerase interactions with the promoter, as these complexes vary in their sensitivity to challenge by heparin. Heparin is a polyanion which competes with DNA for binding to RNA polymerase. The RP_c

complex is sensitive to challenge by heparin, while the RP_i complex is heparin-resistant. Additional intermediates have also been proposed based on temperature-dependent studies on binding and initiation kinetics, and include an additional step between open and initiated complexes, and a "preclosed complex" (Duval-Valentin and Ehrlich, 1987).

In addition, footprinting analysis of RNA polymerase interaction with the promoter region have helped to further define the kinetic intermediates of transcription initiation. For example, there is a dramatic difference in the DNase I pattern of cleavage for the closed and open complexes. Downstream contacts between RNA polymerase and promoter DNA, as well as melting of the DNA helix from approximately -10 to +2, a prerequisite for initiation of mRNA synthesis, are observed with the open complexes.

Sequence-dependent DNA curvature. Initiation of transcription is also affected by the presence of intrinsically curved DNA upstream from the RNA polymerase binding site. Intrinsically curved DNA refers to sequence-dependent DNA curvature, characterized by runs of adenines (A tracts) repeated in phase with the DNA helical repeat, approximately 10.5 bp/turn. This places the recurrent A tracts along the same side of the double helix, so that small bends associated with the A tracts add constructively to generate a large regional curvature. The most striking feature of DNA containing A tract curvature is its altered electrophoretic mobility. Indeed, this has become the hallmark of curved DNA molecules. When electrophoresed on a

polyacrylamide gel, curved DNA molecules display aberrant mobility and migrate more slowly than expected on the basis of their size (Challberg and Eglund, 1980; Simpson, 1979). The degree of electrophoretic anomaly can be reduced (*i.e.*, mobility approaches normal) by increasing the temperature of the gel, by the addition of the antibiotic distamycin, which binds to the minor groove of DNA thereby straightening out regions of curvature, or by interrupting runs of contiguous adenines with another base. Significant DNA bending requires a minumum of four contiguous adenine residues, with the maximum curvature being observed with six adjacent adenines. The magnitude of electrophoretic retardation is also affected by the position of the curved region on the DNA fragments, with mobility most dramatically affected when the curvature is located in the center of the DNA fragment.

The mechanism by which A-tracts bend the DNA is still debated. A conspicuous feature of homopolymers of dA-dT is the high propeller twist, which optimizes base stacking interactions and narrows the width of the minor groove. The minor groove becomes increasingly compressed from the 5' to 3' direction of A tracts. Thus the two ends of an A tract do not contribute equally to curvature. In addition, the base pairs immediately adjacent to A tracts can also influence the degree of curvature, with the greatest effect observed when a C flanks the 5' side and a T flanks the 3' side.

Several theoretical models have been proposed to explain the ability of a DNA sequence to confer intrinsic curvature on the axis of the DNA double helix. The unifying theme among all these models is the hypothesis that DNA bending requires a difference in base pair inclination between the tract of DNA curvature and that of

normal DNA sequence. Initially, models for DNA curvature were proposed in order to explain how DNA is bent for packaging into nucleosomes. One of the early models formulated to explain the phenomenon of DNA curvature was proposed by Trinov and Sussman (Trinov and Sussman, 1980). This model, called the "wedge model", assumes a B-DNA conformation throughout the helix. According to the wedge model, intrinsic DNA curvature is induced by a tilt and roll component between adjacent A-T base pairs. This combination of tilt and roll causes formation of a wedge or angle between adjacent dA-dT base pairs in the DNA helix, which results in a smooth deformation of the DNA axis. The poly A tracts are placed along the inside of the curve, within the compressed minor groove.

Another early model, the junction model (Wu and Cruthers, 1984), predicted that A tracts assume a non- B-DNA conformation, in which curving is due to the abrupt change in DNA conformation at the junction of B-form DNA and the A tracts. The sharp change in conformation at the junction of the two forms was attributed to differences in the angular orientations of the base pairs relative to their respective axes. A popular model at the present time predicts that short tracts of the homopolymer dA-dT adopt a DNA conformation (B') that is distinct from the normal B form DNA. This model predicts that base stacking interactions are the dominant force in the formation and stabilization of the B' form structure (Diekmann, *et al.*, 1992). This group points to the high propeller twist of the dA-dT base pairs as the factor which optimizes base stacking interactions and narrows the minor groove of curved DNA. They also suggest

that the spine of hydration in the minor groove of the B' tracts contributes minimally to DNA curvature as compared to base stacking interactions.

Another model for DNA curvature (Crothers, *et al.*, 1990), attributes extensive hydration of the minor groove as the inducing and stabilizing factor which leads to the negative base pair inclination of A tracts characteristic of curved DNA. This model predicts that removal of the spine of hydration in the minor groove leads to a concomitant decrease in curvature. It further suggests that GC base pairs do not lend themselves as candidates for DNA curvature because the guanosine 2-amino group protrudes into the minor groove, thus interfering with formation of a network of hydration.

Intrinsically curved DNA was discovered through the examination of kinetoplast (mitochondrial) minicircle DNA from the trypanosome *Leishmania tarentolae*, in which phased repeats of A tracts are prevalent (Challberg and Eglund, 1980; Simpson, 1979). Upon electrophoresis in polyacrylamide gels, the kinetoplast minicircle DNA displayed aberrant electrophoretic mobility. Since then, the existence of sequence-dependent curvature of DNA is well-documented in both prokaryotic and eukaryotic genomes. Curved DNA is often associated with DNA structured by DNA binding proteins in a wide variety of biological processes, including replication, transcription, recombination, and chromatin organization. Intrinsically curved DNA affects protein binding to several regulatory regions, including the origins of replication of plasmids pBR322 (Trifanov, 1985), pT181 (Koepsel and Kahn, 1986), and pR6K (Mukherjee, *et al.*, 1985), the bacteriophage λ (Zahn and Blattner, 1985), and ϕX174 (Trifanov, 1985), a yeast

autonomously replicating sequence (Snyder, *et al.*, 1986), Simian virus 40 (Ryder, *et al.*, 1986), and mouse satellite DNA (Trifanov, 1986). In addition, curved DNA has functional implications in recombination, as curved DNA is often associated with IHF binding sites (Kur *et al.*, 1989), and in chromatin organization, where histone wrapping around the octamer core is dependent on a curved sequence motif (Trifonov and Sussman, 1980).

Studies on the role of curved DNA in transcription are well-documented. Curved DNA upstream of the -35 region has been demonstrated to be necessary for a high rate of transcription in both prokaryotes and eukaryotes. Genetic studies on promoters associated with upstream curved DNA have demonstrated a strong correlation between promoter strength and the presence of upstream cuvature (Lamond and Travers, 1983; Bossi and Smith, 1984; Galas *et al.*, 1985; Gourse *et al.*, 1986; Plaskon and Wartell, 1987; Kuhnke *et al.*, 1987, McAlister and Achberger, 1988). Promoters containing upstream curvature are generally associated with highly expressed genes. Furthermore, sequence-dependent DNA curvature has been demonstrated to stimulate transcription when used to replace the upstream binding sites for several transcriptional activator proteins (Gartenburg and Crothers, *et al.*, 1991; Bracco *et al.*, 1989). It is therefore tempting to speculate that protein-mediated DNA bending may be functionally analogous to sequence-dependent DNA curvature.

The presence of in phase polyadenine tracts have been found upstream of several *E. coli* promoters, including the *omp F* promoter (Mizuno, 1987), the *his* promoter and the *lpp* promoter (Verde, *et al.*, 1981), and a subset of rRNA promoters (Brosius *et al.*,

1981) and tRNA promoters (Lamond and Travers, 1983; Bossi and Smith, 1983, 1984; Nishi and Itoh, 1986). Deletion analysis of the upstream curved DNA has suggested that promoter activation is intimately related to sequence dependent curvature. The apparent ubiquity of inherently curved DNA sequences in living organisms, combined with the observation that such motifs are often associated with gene regulatory regions, suggests that there is a linkage between DNA curvature and biological function.

Although DNA upstream of the -35 region has been demonstrated to be necessary for a high rate of transcription from numerous bacterial promoters, upstream curvature appears to be a more highly conserved and common feature among promoters of Gram-positive bacteria such as *Bacillus*. Intrinsically curved DNA upstream of the RNA polymerase binding site strongly influences transcription in *B. subtilis*. Deletions which reduce the upstream A tracts dramatically reduced expression from several promoters in both *E. coli* and *B. subtilis*. For example, initiation of transcription of the *E. coli* galactose P1 promoter was dramatically reduced when regions of upstream DNA curvature were deleted (Bracco *et al.*, 1989). The drugs distamycin and neotropsin serve to remove DNA curvature. The addition of these drugs to linear fragments containing the *gal* P1 promoter and its associated curved DNA elements resulted in a significant decrease in transcriptional initiation in the absence of the cAMP-CAP complex (Lavigne, *et al.*, 1991). These results were taken as strong evidence of the importance of DNA curvature in regulating gene expression from the *gal* P1 promoter. Similarly, deletion mutants in which upstream AT-rich regions have been removed from several *B. subtilis* promoters (e.g., promoters for *spoVG*, *veg*, etc.)

have been found to severely reduce *in vitro* transcription. In fact, it has been postulated that lack of this region may constitute one reason why many strong *E. coli* promoters are poorly recognized by the major *B. subtilis* RNA polymerase.

Recently, Ross *et al.*, (Ross *et al.*, 1993) have proposed that an A-T rich region immediately upstream of the *E. coli rrnBP* promoters, (called the UP element), stimulates transcription by providing specific binding sites for the α subunit of RNA polymerase. Transcriptional fusions between the UP element and other promoters (for example, the *lacUV5*) also stimulated transcription. Mutations in the carboxy terminus of the α subunit of RNA polymerase prevented stimulation of transcription for promoters associated with the UP element. In addition, purified α was shown to preferentially bind to the UP element. These results demonstrated that α plays a direct role in promoter recognition and suggests that the UP element may represent a third recognition element in bacterial promoters (in addition to the -10 and -35 conserved regions). A cautionary statement must be made, however, regarding interpretation of these results. At no time does Ross *et al.*, indicate that the A-T rich UP element is curved. Thus, what effect, if any, UP element secondary structure has on *rrnBP* promoter function can not be determined from this paper.

In an attempt to correlate DNA curvature with biological significance, recent studies have been conducted to search for *E. coli* proteins which preferentially bind curved DNA. First discovered in 1977, (Varshavsky *et al.*, 1977), the *E. coli* nucleoid protein H-NS has since been identified as a protein which preferentially binds regions of curved DNA. The H-NS protein associates with curved DNA sequences with high

affinity *in vitro*, as determined by its ability to preferentially bind a synthetic curved DNA sequence in the presence of an excess amount of non-curved competitor DNA using gel retardation analysis. In addition, removal of the curvature by addition of the DNA intercalating agent distamycin, inhibited binding of the H-NS protein (Yamada, *et al.*, 1990).

Mutations in the *hns* locus are highly pleiotropic, affecting expression of a variety of unrelated genes. In *hns* deletion backgrounds, expression from many promoters is quite divergent, activity may be enhanced or repressed, while still others show little change in expression relative to wild-type cells. Yoshida, *et al.*, (Yoshida *et al.*, 1993), identified several genes located adjacent to the *E. coli trp ABCDE* operon which encode a subset of proteins whose expression is affected by H-NS. The promoters of these genes were used to construct promoter-*lacZ* transcriptional fusions. The effect of H-NS on transcription on these genes was then examined. It was found that expression of some genes was activated in an *hns*-deletion mutant while others were repressed, and yet others remained unaffected. In addition, it has recently been shown that H-NS acts as a repressor of its own transcription. The promoter for H-NS is associated with upstream curvature, and it has been suggested that binding of the H-NS protein to this region is responsible for repression (Ueguchi, *et al.*, 1993). Such results have led to the hypothesis that the H-NS protein may function as a global transcriptional regulator by direct association with curved regions of DNA upstream of the promoters for target genes (Yamada, *et al.*, 1991). It is likely that *E. coli* contains other curved DNA-binding proteins which are less abundant than H-NS. These findings lend support

to the notion that DNA curvature is intimately associated with biological function, perhaps through the formation of higher nucleoprotein complexes, thereby structuring the DNA in such a way as to alter polymerase-promoter contacts. It has been suggested that DNA curvature may function in establishing critical protein-DNA or protein-protein interactions which could facilitate transcriptional initiation, thus stressing the importance of the dynamics of DNA architecture to its function (Wu and Crothers, 1984).

The major RNA polymerases of *E. coli* and *B. subtilis* have similar subunit composition and recognize promoters that have the same -10 and -35 consensus sequences. However, the two polymerases display distinct promoter preferences, with the *B. subtilis* enzyme requiring additional DNA sequences for efficient promoter utilization.

In our lab we have identified promoters dependent on curved DNA upstream of the -35 region. Deletion of this DNA decreased promoter function dramatically when assayed *in vivo* and *in vitro* (McAlister and Achberger, 1988). We have previously shown that optimal positioning of curvature relative to the promoter is essential for efficient promoter utilization. Briefly, short DNA insertions were used to change the distance between the curvature and the -35 binding site. This simultaneously changed two parameters, those being the linear placement and rotational orientation of curved DNA relative to the -35 promoter region. Relative to the original promoter, the most efficient promoters contained insertions which retained the original rotational orientation of the upstream DNA relative to the -35 region. Insertions that altered the orientation reduced the affinity for RNA polymerase (McAlister and Achberger, 1989). There

have been two major models proposed to explain how curved upstream DNA affects binding of RNA polymerase. The first model predicts direct contact between the curve and the RNA polymerase. The second model predicts no direct contact between the curve and the polymerase, but rather, the curve serves to wrap or loop around the DNA and provide additional polymerase-promoter contacts. However, these additional protein-DNA contacts may not necessarily be within the curved DNA region. Although neither model has been ruled out, results from the rotational displacement studies lend support to the second model.

Since the major RNA polymerases from *B. subtilis* and *E. coli* recognize the same -10 and -35 promoter consensus sequences, and since gene expression from *Bacillus subtilis* promoters is highly affected by the presence of upstream A tracts, it was of interest to determine if these promoters would demonstrate the same dependency when utilized in *E. coli*. The present study has examined the ability of curved DNA upstream of the -35 region to influence initiation of transcription in *E. coli* and *B. subtilis*.

EXPERIMENTAL PROCEDURES

Bacterial strains and plasmids. *E. coli* JM83 (Messing, 1979), *E. coli* THK30 and THK31 (Kawula and Orndorff, 1991), and *B. subtilis* 1A510 (Ostroff and Pene, 1984), were used as the experimental hosts. RNA polymerase was isolated from *E. coli* MRE 600 (ATCC #29417) and *B. subtilis* 168 (laboratory culture collection). The relevant genotypes of the bacterial strains used in this study are listed in Table 1.

Table 1. Bacterial Strains

Strains	Relevant Characteristics
E. coli	
JM83	*ara*, Δ(*lac-proAB*), *rpsL*, ϕ80*dlacZ*Δ*M15*
THK30	*pilG2* - *tetR* (null allele) insertion in *pilG*
THK31	*pilG*$^+$ (parental allele) with adjacent insertion of *tetR* gene
MRE600	lacks RNase I
B. subtilis	
1A510	*leuA8*, *arg15*, *thrA*, *recE4*
168	*trp*C2

Plasmids pUC19 and pDMP16 were used for all recombinant DNA work. Plasmid pDMP16 (Pawlyk, 1986) contains the origins of replication for *E. coli* and *B. subtilis*,

and was used as a shuttle vector. In addition, plasmid pDMP16 was the DNA source for the promoterless *cat* gene used in the construction of the λ-derived promoters subcloned into pUC8 (see below).

Media, growth conditions and DNA transformations. All *E. coli* and *B. subtilis* strains were routinely grown in LB medium (Miller, 1972) at 37° C. Liquid cultures were aerated vigorously. For solid media, 1.5% (w/v) agar was added.

Transformation of all *E. coli* strains was accomplished using $CaCl_2$-treated cells as follows (Lederberg and Cohen, 1974). A 1 ml aliquot of an overnight broth culture of *E. coli* was used as an inoculum into 50 ml of LB. Cells were grown at 37° C with shaking aeration until an OD_{600} of 0.5 was reached. Cells were harvested by centrifugation at 7,000 x g for 7 minutes. The supernatant fraction was discarded and the cell pellet was suspended in 40 ml cold 0.1 M $MgCl_2$. The cells were pelleted again by centrifugation at 7,000 x g for 7 minutes. As before, the supernatant fraction was discarded. These cells were then suspended in 20 ml of cold 0.1M $CaCl_2$ and incubated on ice for 20 minutes, then pelleted as before. Finally, the cells were suspended in 2.5 ml cold $CaCl_2$. Competent *E. coli* cells (0.2ml) were incubated with 20-200 ng plasmid DNA, incubated on ice for 20 minutes, and then heat shocked at 42° C for 2 minutes. These cells were briefly cooled on ice followed by the addition of 3 ml of L broth and incubation with shaking at 37° C for 60-90 minutes. Between 0.05 - 0.2 ml of cells were spread onto the appropriate solid medium to select for plasmid containing cells. *E. coli* cells transformed with the shuttle vector pDMP16 were

selected on plates containing 20 μg/ml chloramphenicol, while cells transformed with pUC8 derivatives were selected on plates containing 50 μg/ml ampicillin.

DNA was transformed into *B. subtilis* 1A510 using naturally competent cells. Naturally competent cells were prepared by inoculation into side arm flasks containing 50 ml 1X MG broth, which contained the following components: 1g $(NH_4)_2SO_4$, 7g K_2HPO_4, 3g KH_2PO_4, 0.5g Na citrate dihydrate, 50 μl 50% w/v glucose (filter sterilized), 200 μl from 10mg/ml solutions of the amino acids arginine, threonine and leucine, 6.4 ml 20% w/v $MgSO_4 \cdot 7H_2O$, 2.5 ml 20% w/v casamino acids and 1 liter of deionized water (Bott and Wilson, 1967). Cell growth was monitored spectrophotometrically by measuring the optical density at OD_{600}, and cells were harvested 2 hours after reaching saturation phase, at which time a 1 ml aliquot of competent cells was transformed with 0.5 μg of pDMP16 derived plasmids. The mixture was grown at 37° C for 60 minutes with vigorous aeration, and subsequently, 0.2 ml was plated on L agar containing 10 μg/ml chloramphenicol and 20 μg/ml neomycin. The plates were incubated overnight at 37° C.

Construction of λp_R and λp_L-derived promoters. DNA restriction fragments containing the λp_R and λp_L promoters were isolated from plasmid pGW7, a pBR322 plasmid derivative containing the phage lambda control region (Deutch *et al.*, 1982). The λp_R promoter was extracted from pGW7 DNA as a 194 bp *Alu*I fragment, while digestion with *Hae*III generated a 352 bp fragment containing the λp_L promoter. These blunt ended fragments were purified and ligated into the *Hin*cII restriction site of pUC8

and subsequently screened by restriction analysis to ensure proper promoter orientation with respect to the restriction sites in the pUC8 vector. The λp_R and λp_L promoters each contain a unique *Hinc*II site, which allows them to be cleaved at the -35 region (Daniels *et al.*, 1983). The isolation, purification and cloning of the phage SP82 promoters Alu156 and Bal129 into plasmid pUC8 was described previously (McAlister and Achberger, 1988). Each of these promoters can also be cut by *Hinc*II at position -33. The presence of a *Hinc*II site located at the same position in all of the promoters allowed the DNA upstream of the Alu156 and Bal129 promoters to be substituted for the analogous regions in the λp_R and λp_L promoters.

To make these promoter constructs, the upstream DNA was removed from the λp_R and λp_L promoters by digestion with *Eco*RI and *Hinc*II. The remainder of the promoter attached to the vector was then purified after agarose gel electrophoresis from low melting point (LMP) agarose. Briefly, 1 μg of digested plasmid DNA was loaded onto a 1.0% LMP gel and electrophoresed at 50 V until the bromphenol blue dye approached the bottom of the gel. DNA was stained with ethidium bromide and viewed on an ultraviolet transilluminator. The bands corresponding to vector DNA with promoter DNA were excised from the gel and placed in a 1.5 ml snap cap tube. After a brief centrifugation to pellet the agarose, 300 μl of TE (10 mM Tris, 1 mM EDTA, pH 8.0) were added to the tube. The mixture was incubated at 70° C for 10 minutes to melt the agarose, and subsequently vortexed. The suspension was then placed at -70° C for 10 minutes, thawed and centrifuged for 1.5 minutes. The supernatant fraction was removed and transferred to a clean 1.5 ml snap cap tube and DNA was

ethanol precipitated (Maniatis, *et al.*, 1982). The DNA recovered was quantitated by densitometric analysis.

The DNA upstream of the Alu156 and Bal129 promoters was purified as *Eco*RI, *Hin*cII restriction fragments from 6% polyacrylamide gels (30:0.5, acrylamide:bisacrylamide). The excised bands containing the DNA fragments were crushed in a 10 ml polypropylene tube. Ten milliliters of Tris-borate-EDTA (TBE) buffer (89 mM Tris, 89 mM boric acid, 2.5 mM disodium EDTA) was added and the tube was incubated at 37° C overnight. The tube contents were then filtered through a 10 ml syringe containing a glass fiber filter in order to remove any residual polyacrylamide. A cation exchange column was prepared by packing siliconized glass wool into the tip of a sterile 1 cc syringe and pouring a 0.1-0.2 cc bed volume of DE52 resin. The column was rinsed with low salt buffer (10 mM Tris, 100 mM NaCl, 1 mM EDTA; [pH 7.8]). The DNA sample was loaded onto the column and the column was then rinsed with 1 ml of low salt buffer. The DNA was eluted from the column with 1ml of high salt buffer (10 mM Tris, 1M NaCl, 1 mM EDTA; [pH 7.8]) and the eluant collected in 0.5 ml aliquots in sterile 1.5 ml snap cap tubes. The contents of each tube were ethanol precipated, and the recovered DNA was quantitated by densitometric analysis. The DNA fragments from the Alu156 and Bal129 promoters were then ligated upstream of the λp_R and λp_L promoters, whose original upstream regions had been deleted. This resulted in hybrid promoters containing the upstream curved elements of the Alu156 or Bal129 promoters and the downstream -10 and -35 sequences of the lambda λp_R or λp_L promoters. The Balp_R promoter contains the upstream

sequence of Bal129 and the -10 and -35 sequence of λp_R, while the Alup_R promoter contains the upstream sequence of Alu156 and the -10 and -35 sequence of λp_R. Likewise, the Balp_L promoter contains the upstream region of Bal 129 and the -10 and -35 region of λp_L, and the Alup_L promoter contains the upstream sequence of Alu156 and the -10 and -35 region of λp_L. In addition, a λp_R-λp_L hybrid promoter was also constructed using the same *Hin*cII cloning strategy as previously described. This hybrid promoter contained the -10 and -35 sequences of λp_L and the upstream sequences of λp_R. All ligations between vector and insert DNA were performed using T4 DNA ligase. All promoter constructions were confirmed by restriction enzyme digestion and polyacrylamide gel electrophoresis.

Construction of transcriptional fusions between lambda-derived promoters and the *cat* gene. Each of the λ-derived promoter-containing DNA fragments was excised from the pUC8 polyclonal site with *Eco*RI and *Pst*I, generating fragments of the following size: λp_R, 216 bp; *Alup*$_R$, 234 bp; *Balp*$_R$, 173 bp; λp_L, 374 bp; *Alup*$_L$, 273 bp; and *Balp*$_L$, 212 bp. Each DNA fragment was separated from the vector by polyacrylamide gel electrophoresis and purified as described above. Each promoter DNA fragment was ligated into plasmid pDMP16 that had been digested with *Eco*RI and *Pst*I. These plasmid constructs transformed into *E. coli* JM83 cells. Transformants containing λ-derived promoters were selected by growth overnight at 37° C on L-agar plates containing 20 μg/ml chloramphenicol.

Plasmid isolation. Small scale isolation of plasmid DNA from *E. coli* was performed by the alkaline lysis method, which is a variation of the method of Birnboim and Doly (Birnboim and Doly, 1979). Isolation of plasmid DNA from *B. subtilis* was similar to that used for *E. coli*, but with the following modifications. After addition of the lysozyme solution, the reaction was incubated for 30 minutes at room temperature followed by heat shock at 45-48° C for 2-4 minutes. Isopropanol was used for the initial precipitation of the nucleic acid. Suspension of DNA in gel elution buffer (Maxam and Gilbert, 1980) followed by ethanol precipitation was used to further purify plasmid DNA. Large scale isolation of highly purified plasmid DNA was accomplished by the alkaline lysis method followed by cesium chloride equilibrium density gradient separation in the presence of ethidium bromide. Plasmid DNA was quantitated by measuring the absorbance A_{260} and by densitometric analysis of photographic negatives of ethidium bromide stained agarose gels using a BioRad model 620 video densitometer.

Expression and detection of chloramphenicol acetyltransferase. The λ - derived promoters were assayed using transcriptional fusions to the *cat* gene of pDMP16 in *E. coli* and *B. subtilis*. The promoter-*cat* gene fusions were transformed into *E. coli* strains JM83, THK30, THK31, and *B. subtilis* 1A510. Cultures were grown in 500 ml side arm flasks containing 50 ml L-broth with chloramphenicol (2μg/ml). Three milliliters of cells were harvested by centrifugation in a microfuge for 30 seconds (at 15,600 x g) exactly 4 hours after leaving exponential growth phase. The break between log phase and stationary growth was determined graphically by

extrapolation from both growth curves. Cells were immediately washed in 1 ml 100 mM Tris and 200 mM NaCl (pH 7.8), and the cell pellets were stored at -70° C. Cell-free extracts were prepared by suspending cells in 0.4 ml 100 mM Tris-HCl (pH 7.8) and 28 μl phenylmethylsulfonylfluoride (PMSF). Cells were lysed by sonic disruption. The lysate was centrifuged for 4 minutes in a microcentrifuge, and the supernatant was transferred to a clean 1.5 ml snap cap tube. Chloramphenicol acetyltransferase (CAT) activity was assayed by the method of Shaw (Shaw, 1975). The chloramphenicol-dependent deacetylation of acetyl coenzyme A was monitored at 412 nm in the presence of 5,5'-Dithiobis-(2) nitrobenzoic acid, (DTNB). Units of CAT activity were determined by the rate of deacetylation using an extinction coefficient of 1.36×10^4 l/mol·cm. Protein concentration was determined using the BioRad Protein Assay (BioRad Laboratories) as specified by the manufacturer. CAT specific activity was expressed as μmol chloramphenicol acetylated per minute per mg protein at 25° C.

RNA polymerase isolation. RNA polymerase was isolated and purified from *B. subtilis* 168 and *E. coli* MRE600, as described by Achberger and Whitely (Achberger and Whitley, 1981). Briefly, cells were lysed by sonication and RNA polymerase was purified by the sequential steps of polyethylene glycol-dextran phase partitioning, ammonium sulfate precipitation, gel filtration chromotography, and DNA cellulose chromotography. The RNA polymerase transcription assay measured incorporation of [^{3}H]UTP into trichloroacetic acid-precipitable material (Spiegelman, *et al*, 1978). The transcription reaction buffer for *E. coli* was 40 mM Tris-HCl (pH

7.9), 10 mM $MgCl_2$ and 200 mM NaCl. For *B. subtilis*, the composition of the transcription buffer was similar, with the exception that 50 mM NaCl was used. Fractions containing RNA polymerase were then concentrated in an Amicon Diaflo 202 UF Cell under 42 psi nitrogen gas. The subunit composition and purity of the polymerase fractions were determined by SDS-polyacryamide gel electrophoresis. The δ subunit of *B.subtilis* RNA polymerase was isolated by dissociating partially purified enzyme fractions in 6 M urea, 25 mM NaCl, 10 mM Tris-HCl, and 2 mM EDTA (pH 7.8), followed by passage over a phosphocellulose column (Whatman) which had been equilbrated with the same buffer (Achberger and Whiteley, 1981). The column effluent containing the δ and α subunits were dialyzed against 15% glycerol, 100 mM NaCl, 10 mM Tris HCl and 1 mM EDTA (pH 7.8). DEAE Sephadex A25 column chromotography (Pharmacia, Inc.) was used to separate the δ and α subunits. The purified δ subunit was used to reconstitute holoenzyme as determined by a transcription titration curve, in which the concentration of δ was varied until maximum inhibition from heterologous *E. coli* phage T7 promoters was obtained.

Nitrocellulose filter binding assay. Promoter-containing DNA fragments containing the λp_L, $Alup_L$, $Alup_R$, or $Balp_R$ promoters were excised from the pUC8 vector by digestion with *Eco*RI and *Pst*I. The λp_R promoter-containing fragment was prepared by digestion with *Eco*RI and *Hin*dIII, while the $Balp_L$ promoter fragment was removed from the vector with *Bam*HI and *Pst*I. These combinations of restriction enzymes were necessary in order to generate DNA fragments that could be resolved on

6% polyacrylamide gels. The relative affinity of RNA polymerase for each of the promoters was determined by a competitive binding assay. In this assay, the promoter-containing DNA fragments compete for limiting RNA polymerase. The promoters for which the RNA polymerase has the greatest affinity are bound at the lowest concentration of RNA polymerase. Equal molar amounts (ie., 1.5 μg of digested plasmid DNA) of each promoter DNA were incubated at 37° C for 5 minutes with various amounts of RNA polymerase in 250 μl of a binding buffer consisting of 40 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$ and 200 mM NaCl for *E. coli* RNA polymerase or 40 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, and 50 mM NaCl for *B. subtilis* RNA polymerase. The RNA polymerase-DNA complexes were collected on a nitrocellulose membrane (0.45 μm, Schleicher and Schuell) by filtration at a rate of 6 ml/min. The filter was rinsed with 1ml of 40 mM Tris, 10 mM $MgCl_2$ and transferred to a clean glass tube. To the filter, 0.5 ml of gel elution buffer (500mM ammonium acetate, 10 mM magnesium acetate, 0.5 mM disodium EDTA, 0.1% w/v sodium dodecyl sulfate) was added to denature the polymerase and elute the DNA. The mixture was incubated at 50° C for 10 minutes with vigorous agitation. The filter was discarded, and the remaining solution was ethanol precipated. The promoter-containing DNA fragments were separated by electrophoresis on a 6% polyacrylamide gel (60:1 acrylamide to bisacrylamide) using TBE buffer and quantitated by densitometric analysis of photographic negatives of ethidium bromide-stained polyacrylamide gels. RNA polymerase binding to individual promoter-containing DNA fragments was expressed as a fraction of input DNA.

Gel retardation analysis. The relative affinity of *E. coli* RNA polymerase for both linear and supercoiled DNA templates was measured by gel retardation analysis. Protein-DNA complexes were resolved on 4% polyacrylamide gels (79:1 acrylamide:bis-acrylamide) with a high ionic strength buffer (25 mM Tris, 95 mM glycine, 0.5 mM EDTA, 2.5% glycerol, pH 8.5) (Ausubel, *et al.*, 1989). Approximately 5 μg of pUC8 plasmid DNA containing the λp_R or λp_L promoters, respectively, was digested with *Eco*RI and *Hin*d III to generate 5' single stranded extensions for end-labeling with Klenow fragment of DNA polymerase I. The promoter DNA was then radiolabeled at the 5' end with Klenow fragment and 30 μCi [α-^{32}P]dATP in a reaction containing 1 μl each of 10 mM cold dCTP, TTP, and dGTP. The reaction was allowed to proceed at room temperature for 30-45 minutes, and the labeled DNA fragments were separated from the vector and unincorporated nucleotides by electrophoresis on a 6% polyacrylamide gel and electrophoresed. Radioactive promoter-containing DNA fragments were excised from the gel and purified as described previously.

The minimal saturating amounts of *E. coli* RNA polymerase were determined to be 1.10 μg of RNA polymerase for λp_R and 0.07 μg of RNA polymerase for λp_L by titrating the end-labeled promoter-containing fragments with various amounts of the enzyme. *E. coli* RNA polymerase was incubated with approximately 10,000 cpm (0.3-0.8 ng) of end-labeled promoter DNA with λp_R or λp_L in the presence of 0 μg, 0.1 μg, 0.25 μg, 0.5 μg, and 1.0 μg of unlabeled specific competitor DNA. In each assay, nonspecific competitor DNA (plasmid pMBSU) plus specific competitor DNA equaled

1 μg. Specific competitors were pUC8-derivatives containing the hybrid promoters Alup_R, Balp_R, Alup_L, or Balp_L. The nonspecific competitor was vector DNA with no promoter DNA inserted. Both supercoiled and linearized forms of specific unlabelled competitors were used in this assay. Incubations were carried out at 37° C for 5 minutes in a binding buffer consisting of 40 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 200 mM NaCl, and 0.6 mM dithiothreitol. Twenty-six percent Ficoll plus 0.1% bromphenol blue (2.5 μl) was added to each sample immediately prior to loading on a polyacrylamide gel which had been electrophoresed at 100 V for 90 minutes in a running buffer consisting of 0.1 M Tris, 0.38 M glycine, and 2 mM EDTA (pH 8.5). After the samples were loaded, gels were electrophoresed at 35 mA until the bromophenol blue reached the bottom of the gel. The gels were then transferred to Whatman 3MM paper and dried for autoradiography. Kodak XAR-R X-Ray film was exposed at -70 C° for 12-24 hours and developed according to manufacturer's specifications. The autoradiograms were quantitated by densitometric analysis.

DNaseI footprint analysis. To detect the specific interaction(s) of *E. coli* RNA polymerase with upstream regions of the hybrid promoters, DNaseI footprinting was used (Schmitz and Galas, 1979). For this analysis, singly end-labeled DNA fragments were prepared for the λp_R, Alup_R, and Balp_R promoters. Approximately 4 μg of pUC8 plasmid DNA containing the promoters was digested with *Hin*dIII to generate 5' single-stranded extensions for end-labeling with the Klenow fragment of DNA polymerase I. The DNA was end-labeled as previously described and subsequently digested with

*Eco*RI to remove the promoter containing region from the remainder of the plasmid. Radioactive promoter fragments were then purified by the crush and soak method described previously. This generated promoter-containing DNA fragments labeled on a single 3' end. This allowed analysis of the upper strand (*i.e.* nontemplate strand) of the promoter.

To minimize the possibility of multiple RNA polymerase molecules binding to a single DNA fragment, the concentration of enzyme needed to give 60-90% of the maximum filter retention was determined. The end-labeled promoter-containing DNA fragments were titrated with various amounts of enzyme using a nitrocellulose filter binding assay. The appropriate amount of RNA polymerase was determined for each of the DNA fragments analyzed by DNaseI footprinting.

DNaseI footprinting analyses were performed in 180 μl reactions containing the *E. coli* RNA polymerase binding buffer (40 mM Tris; pH 7.9, 10 mM $MgCl_2$, 200 mM NaCl), 30,000 cpm of singly end-labeled promoter-containing DNA, and *E. coli* RNA polymerase. The protein-DNA complexes were incubated for 5 minutes at 37° C. DNaseI digestion was carried out by the addition of 10 μl of a 1:30 dilution of DNaseI [2 mg/ml] to the reaction mixtures, followed by incubation for 45 seconds at 37° C. After this time, 20 μl of a salt solution (19 μl of 3M Sodium acetate and 1 μl tRNA [1mg/ml]) was added to the reactions. This was immediately followed by the addition of 200 μl of phenol to stop the digestions. The mixture was vortexed vigorously and placed on ice for 5 minutes, vortexed again, and centrifuged in a microfuge for 2 minutes. After centrifugation, the top aqueous layer containing the DNA was

transferred to a new snap cap tube and the phenol was removed by the addition of 500 μl of water-saturated ether. The tubes were inverted and the layers were allowed to separate. The top aqueous layer was discarded and the process of ether addition was repeated. Any remaining ether was removed by leaving the samples exposed under a laminar flow hood for approximately 10 minutes. The samples were then ethanol precipated for 15 minutes at -70 °C, followed by centrifugation for 15 minutes in a microcentrifuge. The supernatant was discarded and the pellet was washed with 70% ethanol, dried, and resuspended in 5 μl of formamide loading buffer (10ml formamide, 10 mg xylene cyanol, 10 mg bromphenol blue, 0.2 ml 0.5 M EDTA, pH 8.0). The samples were heated for 5 minutes at 90° C, immediately transferred to ice, and loaded onto a 6% (30:1.5, acrylamide:bisacrylamide) polyacrylamide gel. After the samples were loaded, sequencing gels were electrophoresed at 1700 V for approximately four hours in TBE running buffer. The gels were transferred to Whatman 3MM paper and dried for autoradiography as before. The autoradiograms were quantitated by densitometric analysis.

A G>A sequencing reaction (Maxam and Gilbert, 1980) was performed by methylation of ^{32}P-end labeled promoter-containing DNA and subsequent cleavage of guanines and adenines. Approximately 70,000 cpm of singly end-labeled promoter DNA was methylated with 1 μl of dimethyl sulfate (DMS) for 30 seconds at 37° C. After this time, 55 μl of stop buffer (50 μl 1.5 M NaOAc; ph 7.0, 10 μl β-mercaptoethanol, 5 μl [1mg/ml tRNA]) and 700 μl of 95% ethanol were added to the reaction mixture. The samples were then placed at 70° C for 15 minutes. The

hydrolysis of methylated purines was accomplished by the addition of 35 μl of 5 mM sodium phosphate; pH 7.2 for 15 minutes at 90° C, followed by the addition of 2 μl of 1.2 M NaOH for 15 minutes at 90° C. The samples were then ethanol precipated and resuspended in 5 μl of urea-dye mix (5M urea, 0.1% bromophenyl blue). Immediately prior to electrophoresis on a sequencing polyacrylamide gel, the samples were heated at 90° C for 5 minutes and quickly chilled on ice.

DNA fragment electrophoretic mobility determinations. To determine the extent of aberrant electrophoretic mobility of the λ-derived promoters, 10% polyacrylamide gels (60:1 monomer to bis ratio) were used. Each promoter was excised from the pUC8 vector with either *Eco*RI and *Hin*dIII, *Eco*RI and *Pst*I, or *Bam*HI and *Pst*I. After ethanol precipitation of the promoter-containing DNA fragments, each sample was electrophoresed at 60 V at 4° C for 48 hours, or 65° C for 6 hours. All gels used a TBE buffer system. A peristaltic pump was used to recycle the running buffer between the upper and lower reservoirs of the gel run at 4° C for 48 hours. The apparent size of DNA fragments was calculated relative to size standards composed of pBR322 digested with *Msp*I.

Materials. DNA restriction and modification enzymes, and Ribonuclease A were purchased from New England Biolabs, Inc. and Bethesda Research Laboratories and were used according to the manufacturer's specifications. HPLC grade deoxyribonucleotides were purchased from Sigma and Boehringer Mannheim. The [^{32}P]

deoxyadenosine 5'-triphosphate was purchased from New England Nuclear, Dupont, and [^{3}H] Uridine 5'-triphosphate was purchased from I.C.N. Ribonucleoside triphosphates were purchased from Sigma Chemical Company. All other experimental reagents were of the highest quality commercially available.

RESULTS

This work examines the ability of DNA curvature upstream of the -35 promoter region to affect the interactions between the RNA polymerase and promoter DNA. The Alu156 and Bal129 promoters from the *B. subtilis* bacteriophage SP82 possess intrinsic DNA curvature upstream of their -35 region, as characterized by in-phase runs of adenines, (A tracts), and are efficiently recognized by the major *B. subtilis* RNA polymerase. The nucleotide sequence immediately upstream of the -35 region for the Alu156 and Bal129 promoters shown in Figure 1. It is important to note that although the nucleotide sequence upstream of the Alu156 and Bal129 promoters are similar, they are not identical, and thus would be predicted to have similar but distinct structures. Previous studies have demonstrated the importance of upstream curvature for efficient utilization of promoters Alu156 and Bal129 in *B. subtilis* (McAlister and Achberger, 1988). Since transcription from *B. subtilis* promoters is greatly affected by the presence of upstream A tracts, it was of interest to determine if these regions of curved DNA could affect transcription from heterologous promoters in *B. subtilis* and *E. coli*. A series of four hybrid promoters was constructed in which curved DNA upstream of the -35 region in the Alu156 and Bal129 promoters was substituted for the analogous region of the lambda promoters λp_L and λp_R. This substitution yielded hybrid promoters which contained the upstream curved DNA of Alu156 or Bal129 fused to the -10 and -35 sequences of the λp_L and λp_R promoters. Hybrid promoter construction was facilitated due to the presence of a *Hinc*II restriction site within the -35 region of all the promoters used in this study. The lambda phage promoters λp_R and λp_L

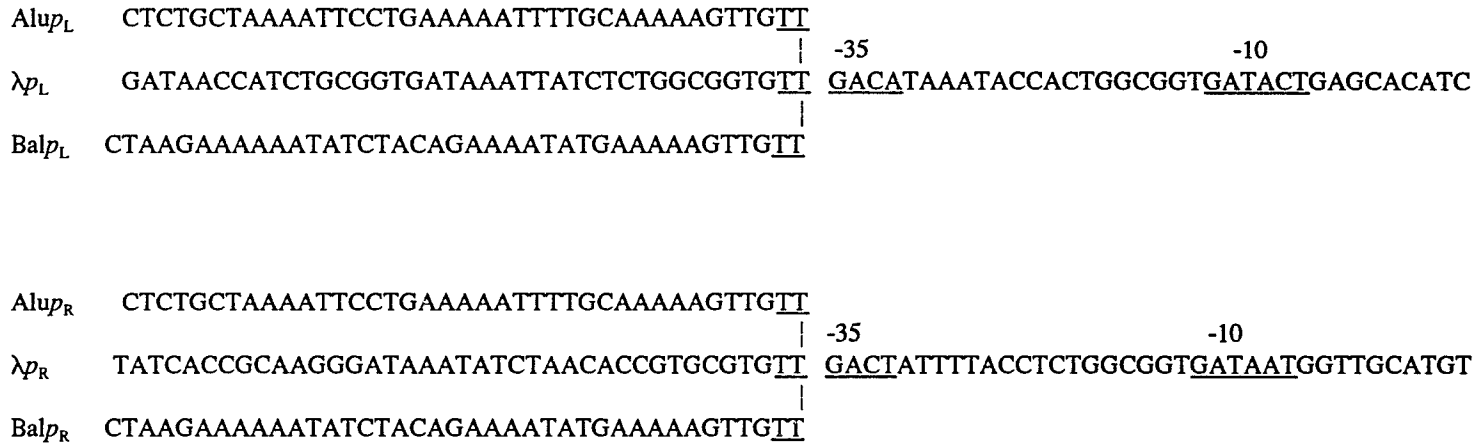


Fig. 1. Nucleotide sequence of the λp_R and λp_L promoters. The nucleotide sequence of the λp_R and λp_L promoters is shown along with the nucleotide sequence of the curved DNA from the *B. subtilis* phage SP82 promoters Alu156 and Bal129 listed above and below, respectively (Daniels, *et al.*, 1983; Panganiban and Whiteley, 1983; McAllister and Achberger, 1988). These curved DNA sequences were used to construct the hybrid promoters in this study. Hybrid promoters with the prefix Alu- contain the curved DNA from Alu156 and hybrid promoters with the Bal- prefix contain the curved DNA from Bal129. The -10 and -35 region of each promoter have been underlined.

are efficiently recognized by the major *E. coli* RNA polymerase, the σ^{70}-containing enzyme. On the basis of homology to the *E. coli* consensus sequence, the λp_R and λp_L promoters have been assigned scores of 58.6% and 58%, respectively (Mulligan, *et al.*, 1984). The two promoters differ by only one nucleotide from each other in their -10 and -35 regions and both promoters have the same 17 base pair spacing between these regions (Daniels *et al.*, 1983). This is an important parameter to consider, as the major RNA polymerases from *E. coli* and *B. subtilis* share the same consensus sequence at the -10 and -35 region.

Electrophoretic mobilities of promoter-containing DNA fragments. The distinguishing physical property of curved DNA is its reduced electrophoretic mobility on polyacrylamide gels. The degree of electrophoretic retardation depends on the positioning and extent of the bent DNA sequence. The original λp_R and λp_L promoters and promoters derived from them were examined for aberrant migration when electrophoresed through high percentage polyacrylamide gels. The results from this experiment are presented in Table 2. The original λp_R and λp_L promoters exhibited similar degrees of aberrant electrophoretic mobility, despite the fact that the λp_L promoter contains more regions of A tracts than λp_R (Daniels *et al.*, 1983). Addition of upstream curvature to the λp_R promoter generated hybrids which significantly changed the DNA fragment mobility, with Balp_R displaying the greatest deviation from expected mobility. The hybrid promoters of the λp_L series exhibited electrophoretic mobilities more in keeping with their size based on nucleotide sequence than did the λp_R

derivatives. The values observed for the λp_R and λp_L promoters are consistent with those obtained by McAlister (McAlister, 1988).

Table 2. Apparent Size and Actual Size for Promoter Containing DNA Fragments

Promoters	Fragment length[a]	Apparent size[b] / actual size
λp_L	374	1.06
Alup_L	273	1.04
Balp_L	202	1.11
λp_R	224	1.07
Alup_R	242	1.16
Balp_R	173	1.24
p_R-p_L	245	0.98

[a] Length in basepairs based on nucleotide sequence determination.
[b] Apparent size determined by using *Msp*1-digested pBR322 as the standard.

The λp_R promoter has a run of thymines (*i.e.*, an A tract on the opposite strand) that is in phase with the curved DNA in the hybrid promoters. This second region of DNA curvature is not present in the λp_L series of promoters. The run of thymines in the λp_R promoter is also absent in the $p_R p_L$ hybrid, as is the upstream region of curvature in λp_L. The $p_R p_L$ hybrid promoter exhibited electrophoretic mobilities which directly corresponded with its size. This is indirect evidence suggesting that the T-tract at -25 in λp_R does contribute to its aberrant electrophoretic mobility. Therefore, results from this analysis suggest that the overall shape due to curvature of the λp_R hybrids is different from that of the λp_L hybrids.

CAT expression of the λ-derived promoter constructs. To examine the influence of upstream DNA on transcription from our hybrid promoters, transcriptional fusions were made between each of the λ-derived promoters and the *cat* gene carried on the shuttle vector pDMP16. Chloramphenicol acetyltransferase (CAT) specific activity was measured for each of the promoter constructs in *E. coli* and *B. subtilis*. The results from this series of experiments are presented in Table 3.

Table 3. Promoter Utilization in *E. coli* and *B. subtilis*

	[a]CAT Specific Activity	
Promoter	*E. coli*	*B. subtilis*
λp_R	0.47 (±0.03)[b]	0.81 (±0.05)
Alup_R	0.34 (±0.02)	0.60 (±0.06)
Balp_R	0.70 (±0.11)	0.51 (±0.11)
λp_L	0.37 (±0.05)	0.10 (±0.01)
Alup_L	0.33 (±0.02)	0.46 (±0.06)
Balp_L	0.72 (±0.12)	0.81 (±0.06)

[a]Specific activity of chloramphenicol acetyltransferase expressed as micromoles chloramphenicol acetylated per minute per milligram protein at 25°C.

[b]Numbers represent averages of between 3-5 each.

Replacement of the upstream DNA of the λp_L promoter with curved DNA was shown to stimulate transcription in *B. subtilis*. The hybrid promoter Balp_L displayed the greatest CAT specific activity when assayed in *B. subtilis*, increasing promoter activity by approximately eight-fold. *B. subtilis* with the Alup_L promoter also demonstrated an

increase in CAT activity, approximately four-fold, as compared to the original λp_L promoter. Cells with the Balp_R promoter displayed a slight reduction in CAT activity as compared with the original λp_R promoter. This result was somewhat surprising, since the same upstream curved sequence significantly enhanced expression from the λp_L promoter. It is possible that the nucleotide sequence of the p_R and p_L promoters may dictate the optimal positioning of DNA curvature. As mentioned previously, the two lambda promoters have similar nucleotide sequences with a few possibly significant differences. In addition, the two regions of curved DNA upstream of the Alu156 and Bal129 promoters would be predicted to have similar but not identical structures. Subtle changes in DNA curvature may result in dramatic changes in promoter function. In *B. subtilis*, transcription from the λp_R and p_L promoters was affected differently by the addition of curved upstream DNA sequences from Alu156 and Bal129. The hybrid p_L promoters displayed a significant increase in CAT specific activity relative to λp_L. In contrast, the λp_R promoter yielded a greater CAT specific activity than either of the curved p_R hybrids. Thus, the addition of curved DNA from Alu156 and Bal129 upstream of the λp_R promoter decreased promoter activity in *B. subtilis*. It is possible that the decreased *in vivo* activities of the Alup_R and Balp_R promoters is due to a tight binding between the polymerase and the promoter which results in reduced promoter clearance. This parallels the findings of McAlister and Achberger (McAlister and Achberger, 1988), who demonstrated the same effect on transcription in *B. subtilis* with the Bal129 and Bal129-Alu156 hybrid promoters. The authors proposed that the upstream curvature in the promoters contributed to a tight binding between the

polymerase and the promoter, which decreased the ability of the polymerase to clear the promoter and engage in productive transcription.

When these same promoter constructs were tested in *E. coli*, the hybrid promoters appeared similar to the original promoters, although a slight increase in CAT activity, approximately two-fold, was observed with the Balp_R and Balp_L hybrid promoters relative to the promoters λp_R and λp_L, respectively. Although the absolute values for CAT specific activity cannot be directly compared between *E. coli* and *B. subtilis*, the relative changes in CAT expression from derivatives of the same promoter series can be compared. Thus, results from this experiment suggest that *E. coli* RNA polymerase is less affected by the presence of upstream DNA curvature than is *B. subtilis*.

Since *E. coli* RNA polymerase appeared to be less affected by the presence of upstream DNA curvature, we considered the presence of proteins in *E. coli* that could mask the effect of curvature on gene expression. The *E. coli* H-NS protein is a sequence-specific DNA binding protein that binds preferentially to curved DNA. The interaction of the H-NS protein with regions of upstream DNA curvature might affect the structure of the DNA in such a way as to alter polymerase-promoter contacts, by either optimizing or inhibiting protein-DNA contacts. To examine the ability of the H-NS protein to influence transcription from our hybrid promoters *in vivo*, CAT specific activity in *E. coli* strains THK 30 and THK 31 was measured for the transcriptional fusions between each of the promoter constructs and the *cat* gene carried on pDMP16. Strains THK30 and THK 31 are isogenic except for the *hns* locus; THK 30 is an *hns*

mutant. The level of CAT specific activity measured for each of the promoter constructs in the *E. coli* THK 30 and THK 31 strains is presented in Table 4.

Table 4. Promoter Utilization in an *E. coli hns* Mutant Strain

Promoters	[a]CAT specific activity	
	THK30 (*hns*-)	THK31 (*hns*+)
λp_R	1.27 (±0.34)[b]	0.47 (±0.16)
Alup_R	0.59 (±0.08)	0.79 (±0.14)
Balp_R	1.86 (±0.58)	0.89 (±0.47)
λp_L	0.25 (±0.01)	0.27 (±0.02)
Alup_L	0.22 (±0.04)	0.23 (±0.02)
Balp_L	0.45 (±0.08)	0.36 (±0.08)

[a]Specific activity of chloramphenicol acetyltransferase expressed as micromoles chloramphenicol acetylated per minute per milligram protein at 25° C.

[b]Numbers represent averages of between 3-5 each.

Within the λp_L series of promoters, there was no influence of the H-NS protein, as there was no significant difference in CAT expression in the THK 30 or THK 31 strains. The results observed in the λp_R promoter series were more difficult to interpret. While the Balp_R and λp_R promoters exhibited an increase in CAT expression in the absence of the H-NS protein, this effect did not correlate with curved DNA upstream of the -35 region. Thus, the H-NS protein in *E. coli* did not appear to be masking the effect of curved DNA or any role it plays in transcription initiation with the λ-derived promoters used in this study.

Relative affinity of RNA polymerase for promoters using the filter binding assay. To determine if the levels of *in vivo* promoter activity, as determined by the CAT assay, reflected changes in the affinity of RNA polymerase for the promoters, a series of promoter competition experiments were performed using a nitrocellulose filter-binding assay. In this assay, equal molar amounts of each of the promoter constructs were incubated at 37° C for 5 minutes in a reaction mixture containing RNA polymerase isolated from either *E. coli* or *B. subtilis*. The reaction then was filtered through a nitrocellulose filter under aspiration. Free double stranded DNA passes through the filter, while DNA-protein complexes are retained. The DNA was recovered from the protein-DNA complexes and electrophoresed on a 6% polyacrylamide gel.

A representative polyacrylamide gel for the analysis of *E. coli* RNA polymerase binding to promoter-containing DNA fragments as a function of enzyme concentration is presented in Figure 2. Each lane of the gel in Figure 2 demonstrates the promoter-containing DNA fragments bound by *E. coli* RNA polymerase at the enzyme amounts listed. At low amounts of RNA polymerase, little binding is observed. As the amount of RNA polymerase is increased, the promoter DNA fragments for which RNA polymerase has the greatest affinity are bound first. In this promoter competitive binding assay, it is possible to determine the relative affinity of RNA polymerase for each of the promoters tested. Absolute measurements are not possible since the promoter sites found on pUC8 are present in seven times the amount of any one of the test promoters. When the test promoters (*i.e.*, λp_L, λp_R, and their derivatives) were

Fig. 2. *E. coli* RNA polymerase binding to promoter-containing DNA fragments as a function of enzyme concentration. Polymerase-bound DNA fragments were extracted from nitrocellulose filters and separated by polyacrylamide gel electrophoresis. The first lane labeled, Control, contains 1 μg of digested plasmid DNA for each promoter. The DNA bands are labeled with the promoter contained on each fragment. *E. coli* RNA polymerase was added to binding assays at the amounts (μg) listed at the top of each lane.

excised from the pUC8 vector, the vector DNA was included in the assay. The excess of vector DNA ensured that binding artifacts, such as DNA fragment end-binding, were not significant in the assay. RNA polymerase retention of vector DNA can be observed at the top of the figure.

The results of binding assays were quantitated densitometrically from photographic negatives of the polyacrylamide gels. The graphs represent the fraction of total promoter DNA bound as a function of RNA polymerase concentration (Figure 3 and 4). Using this analysis, RNA polymerase binding to each promoter DNA fragment was quantified. Only the most stable promoter-enzyme complexes, (e.g., open complexes), can be measured by the filter-binding assay. Promoter-enzyme complexes with half-lives shorter than the filtration time can not be detected by this assay. In Figure 3B, *E. coli* RNA polymerase displayed a greater affinity for the λp_R-derived hybrid promoters, Alup_R and Balp_R, than for the original λp_R promoter. Indeed within the λp_R promoter series, *E. coli* RNA polymerase demonstrated the strongest binding affinity for the Alup_R promoter. The three λp_L series promoters displayed no significant differences in binding with *E. coli* RNA polymerase (Figure 3A). Since the λp_L promoter itself contains some inherent DNA cuvature upstream and binds *E. coli* RNA polymerase very efficiently, the p_R-p_L promoter was constructed to provide a "non-curved" version of the λp_L promoter with which to compare curved λp_L hybrid constructs. Based on sequence analysis, no intrinsic DNA curvature was noted with the λp_R promoter's upstream DNA. When tested, the λp_R-λp_L hybrid promoter demonstrated a reduced affinity for binding *E. coli* RNA polymerase as compared to

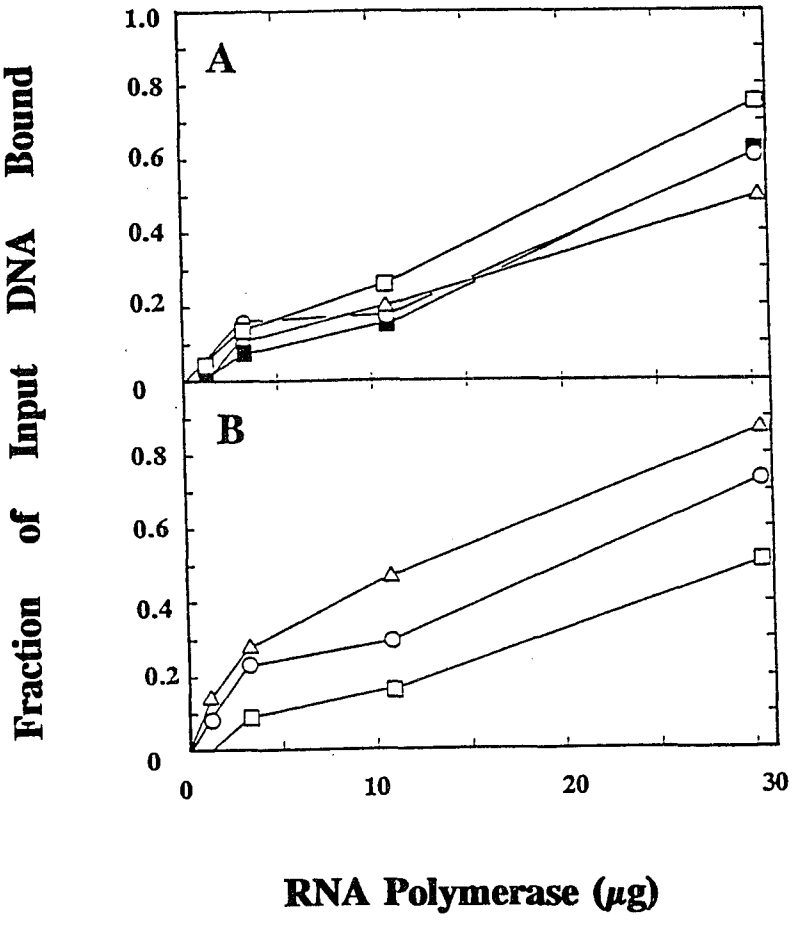


Fig. 3. Relative affinity of *E. coli* RNA polymerase for the promoters λp_R and λp_L and their derivatives. The competition binding assay measured the nitrocellulose filter retention of the promoter-containing DNA fragments as a function of *E. coli* RNA polymerase concentration. At each RNA polymerase concentration, the binding reaction contained equal molar amounts of DNA fragments containing the λp_L (□), Alup_L (Δ), Balp_L (○), $p_R p_L$ (■) promoters (panel A), or the λp_R (□), Alup_R (Δ) and Balp_R (○) series of promoters (panel B).

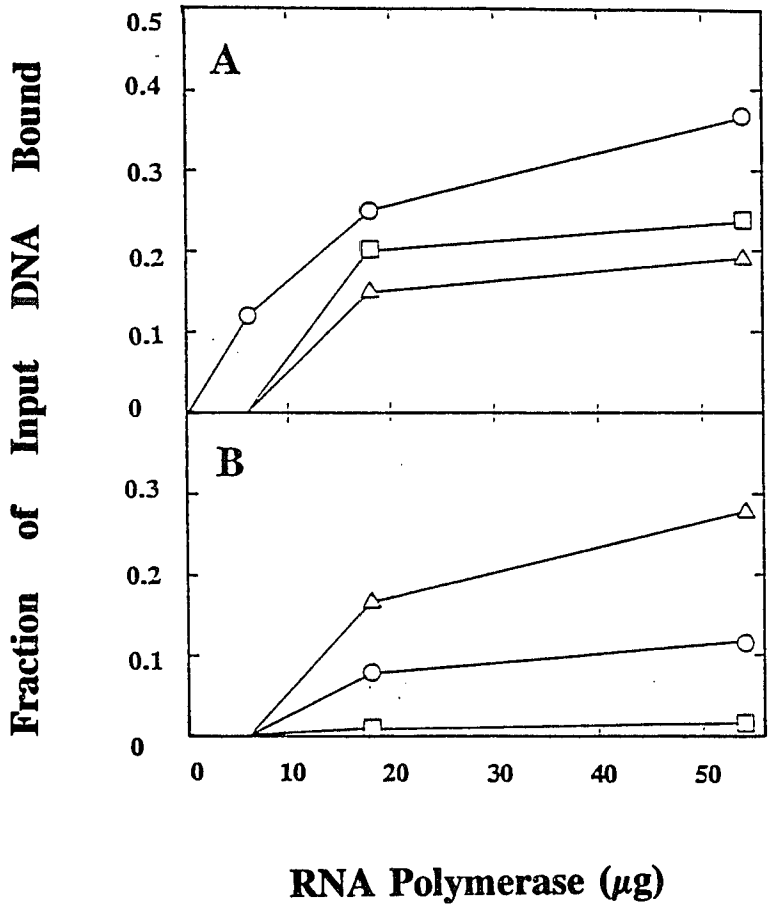


Fig. 4. Relative affinity of *B. subtilis* RNA polymerase for λp_R and λp_L original and hybrid promoters. The nitrocellulose filter retention of the promoter-containing DNA fragments was measured as a function of *B. subtilis* RNA polymerase concentration. At each RNA polymerase concentration, the binding reaction contained equal molar amounts of DNA fragments containing the promoters λp_L (□), Alup_L (Δ), or Balp_L (○) (panel A). Panel B corresponds to binding reactions which contained the promoters λp_R (□), Alup_R (Δ) or Balp_R (○).

the original λp_L (Fig. 3A). However, *E. coli* RNA polymerase binding to the p_R-p_L promoter was still comparable to the hybrid promoter containing curved DNA at the higher RNA polymerase concentrations.

When the same promoter fragments were tested for binding by RNA polymerase from *B. subtilis*, the λp_R hybrid promoters were bound much more efficiently than the original λp_R promoter, (Figure 4B). The *B. subtilis* RNA polymerase demonstrated the strongest affinity for the Alup_R hybrid promoter. Binding to the original λp_R promoter was barely detectable even at relatively high levels of *B. subtilis* RNA polymerase. This would be consistent with the formation of few open promoter complexes or very transient open promoter complexes.

The affinity of *B. subtilis* RNA polymerase for the Alup_L promoter was comparable to that for the original λp_L promoter (Figure 4A). Overall, the *B. subtilis* RNA polymerase bound the λp_L series of promoters more effectively than the λp_R series. In the λp_L promoter series (Figure 4A), the Bal λp_L promoter clearly displayed the strongest binding affinity for RNA polymerase from *B. subtilis.*

In general, *B. subtilis* RNA polymerase bound hybrid promoters on linear templates more effectively than the original promoters. *E. coli* RNA polymerase demonstrated a greater binding preference for the λp_R series of hybrid promoters than the λp_R promoter. Generally, although *E. coli* RNA polymerase does not appear to be as selective in differentiating between promoters associated with curvature as does *B. subtilis,* results from this series of experiments suggest that curved DNA can affect

binding of both *E. coli* and *B. subtilis* RNA polymerase to heterologous promoters on relaxed, linear templates.

Gel retardation analysis of the relative affinity of RNA polymerase for linear and supercoiled molecules. It has been reported that replacement of the CAP site on the *lac* promoter with curved DNA functionally substituted for CAP-cAMP activation of this promoter, but only with negatively supercoiled templates (Gartenberg and Crothers, 1991). It was hypothesized that the DNA supercoiling may affect the binding to hybrid promoters with curved DNA. A series of competition assays were performed to determine the relative affinity of *E. coli* RNA polymerase for hybrid promoters on supercoiled and linearized templates. *E. coli* RNA polymerase binding to end-labeled promoter DNA fragments containing either λp_R or λp_L was measured as a function of supercoiled or linear competitor DNA concentration using gel retardation analysis. This assay is based on the observation that protein-DNA complexes migrate more slowly through non-denaturing polyacrylamide gels than do unbound DNA fragments. As the concentration of the specific competitor (*i.e.*, DNA containing one of the promoters) increases, the band representing the RNA polymerase complex to the end-labeled promoter will decrease. The greater the affinity of RNA polymerase for a specific competitor promoter, the greater the reduction in the band corresponding to the RNA polymerase complexed with the end-labeled promoter-containing DNA fragment.

Figures 5, 6 and 7 correspond to representative autoradiograms from the gel retardation analysis. All reactions contained a constant amount of ^{32}P end-labeled

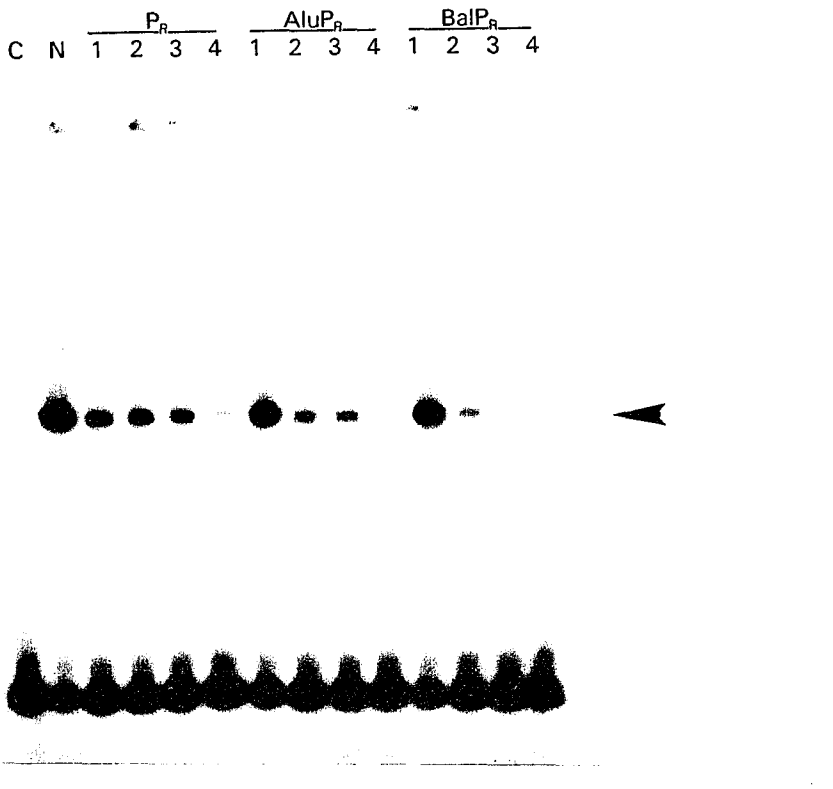


Fig. 5. Use of the gel retardation assay to measure binding of *E. coli* RNA polymerase to λp_R in the presence of supercoiled DNA competitors. End-labeled DNA fragment containing the promoter λp_R was added to various amounts of unlabeled supercoiled competitor DNA. The DNA mixture was incubated with 0.75 μg of *E. coli* RNA polymerase at 37° C for 5 minutes, followed by electrophoresis. All reactions (except lane C), contained *E. coli* RNA polymerase. Lane C is a control lane and represents unbound λp_R. Lane N corresponds to 1 μg non-specific competitor DNA (pMBSU). Lanes 1, 2, 3, and 4 correspond to 0.1 μg, 0.25 μg, 0.5 μg, and 1.0 μg, respectively, of specific competitor DNA λp_R, Alup_R and Balp_R. The arrow indicates the band observed when *E. coli* RNA polymerase binds to the promoter.

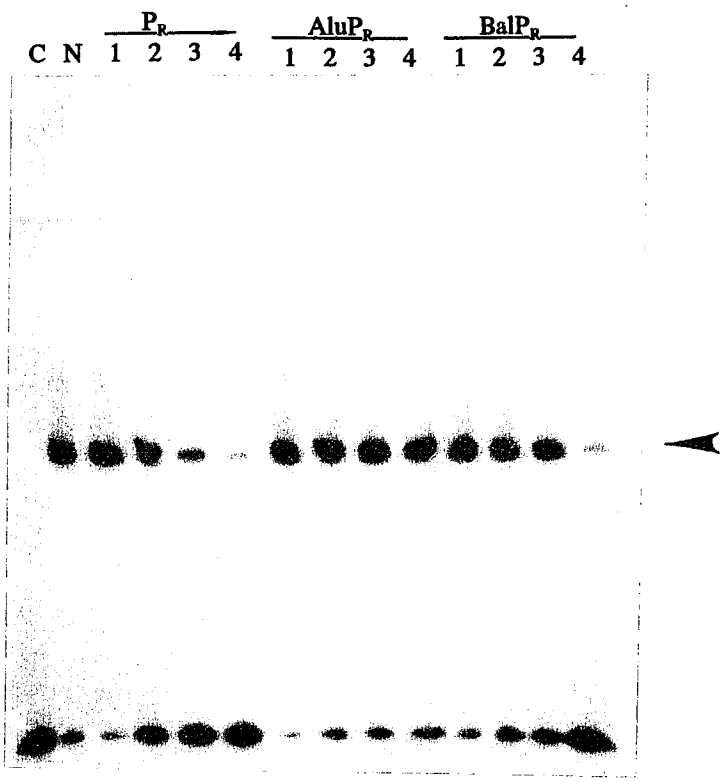


Fig. 6. Binding of *E. coli* RNA polymerase to λp_R in the presence of relaxed specific competitor DNA. Gel retardation analysis was used to indirectly determine the relative binding affinity of *E. coli* RNA polymerase for specific competitors λp_R, Alup_R, or Balp_R. All specific competitor DNAs were added in a linearized, relaxed form. End-labeled promoter fragment λp_R was added to various amounts of unlabeled, competitor DNA. The DNA mixture was incubated with 0.75 μg of *E. coli* RNA polymerase at 37° C for 5 minutes, followed by electrophoresis. Binding reactions contained 1 μg of DNA total, (specific and nonspecific competitor DNA). Reactions were performed with ^{32}P end-labeled p_R without *E. coli* RNA polymerase (lane C), with 1 μg non-specific competitor DNA pMBSU (lane N), and with specific competitor DNAs. Lanes 1,2,3 and 4 correspond to 0.1 μg, 0.25 μg, 0.5 μg and 1.0 μg, respectively, of specific competitor DNA λp_R, *Alup*$_R$ and *Balp*$_R$. The arrow indicates the location of the promoter λp_R-RNA polymerase complex.

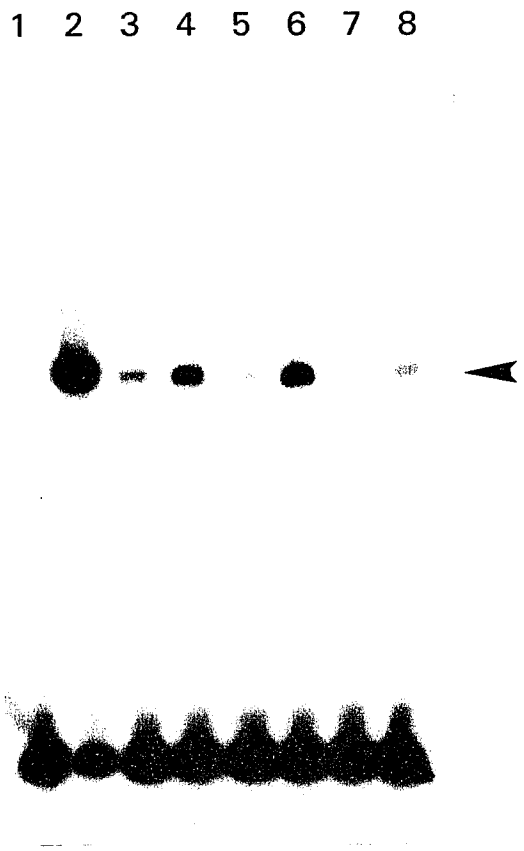


Fig. 7. *E. coli* RNA polymerase binding to λp_L in the presence of supercoiled and relaxed competitor DNA. End-labeled DNA containing the λp_L promoter fragment was added to 1 μg of either supercoiled or linear competitor DNA. The DNA mixture was incubated with 1.05 μg of *E. coli* RNA polymerase at 37° C for 5 minutes, followed by electrophoresis. All reactions (except for lane 1) contained *E. coli* RNA polymerase. Reactions were performed with ^{32}P end-labeled p_L without *E. coli* RNA polymerase (lane 1), with 1 μg non-specific competitor DNA pMBSU (lane 2), and with specific competitor DNA. Lanes 3, 5, and 7 correspond to 1 μg of supercoiled specific competitor DNA with λp_L, Alup_L and Balp_L, respectively. Lanes 4, 6, and 8 correspond to 1 μg of linear specific competitor DNA with p_L, Alup_L, and Balp_L, respectively. The arrow indicates the complex of *E. coli* RNA polymerase and end-labeled λp_L.

promoter fragment λp_R or λp_L. When a specific competitor DNA was used, the amount of nonspecific DNA was decreased such that there was always 1 μg of unlabeled DNA in each assay. The competitions were performed by incubating various amounts of unlabeled specific competitor DNA with end-labeled promoter fragments, followed by the addition of *E. coli* RNA polymerase. Products were resolved by gel electrophoresis. Bands observed on the autoradiogram corresponded to the fast migrating free DNA or the slow migrating complex observed when labeled promoter DNA is bound by *E. coli* RNA polymerase. A decrease in the intensity of the slow migrating band indicates that the unlabeled competitor DNA effectively competed for the RNA polymerase. In Figure 5, the end-labeled promoter DNA was λp_R, and all competitor DNAs were supercoiled. All lanes of the gel, except for lane C, contain 0.75 μg of *E. coli* RNA polymerase. Lane C is a control lane and contains only the labeled DNA. Lane N demonstrated the binding of RNA polymerase in the presence of nonspecific competitor DNA. The specific competitor DNAs used in the competition binding are indicated above each lane. In each case, lanes 1, 2, 3 and 4 correspond to 0.1 μg, 0.25μg, 0.5μg and 1.0 μg, respectively, of the competitor DNAs listed. The intensity of the slow migrating band is dramatically decreased by the addition of Balp_R at 0.5 μg and 1 μg. This indicates that in supercoiled forms, Balp_R is the most effective of the λp_R series of promoters in competing for binding to *E. coli* RNA polymerase. Supercoiled Alup_R was less effective in binding *E. coli* RNA polymerase than Balp_R, but more efffective than the original λp_R promoter.

An analogous experiment using linearized competitor DNA is presented in Figure 6. The location of the free DNA and shifted RNA polymerase labeled DNA complex are comparable to those in Figure 5. The linear competitor DNAs, most notably Alup_R, are much less effective in binding RNA polymerase than their supercoiled form in Figure 5. Densitometry was used to quantify the labeled complex in each lane.

Competition by the λp_L series of promoters for binding to *E. coli* RNA polymerase is presented in Figure 7. The end-labeled promoter in this assay is λp_L. Specific competitors were added in either supercoiled or linear form at a concentration of 1 μg each. Lane 1 is a control lane and contains only end-labeled λp_L. Lane 2 represents the addition of 1 μg of nonspecific competitor DNA. Lanes 3, 5 and 7 correspond to the addition of 1 μg of supercoiled specific competitor DNA with λp_L, Alup_L and Balp_L, respectively. Lanes 4, 6 and 8 correspond to 1 μg of λp_L, Alup_L and Balp_L, respectively, in linear form. In both supercoiled and linear form, the Balp_L promoter was the most effective in competing for binding to *E. coli* RNA polymerase. Alup_R was more effective in binding *E. coli* RNA polymerase than the original λp_L promoter.

The results of the gel retardation assay for the λp_L series of promoters is presented in Figure 8. In general, the promoter-containing DNA competitors were much more effective (*i.e.*, bound the RNA polymerase more efficiently) when supercoiled (Figure 8B) than when the same plasmid DNAs were relaxed by restriction digestion (Figure 8A). In either form, plasmid DNA containing the hybrid promoters

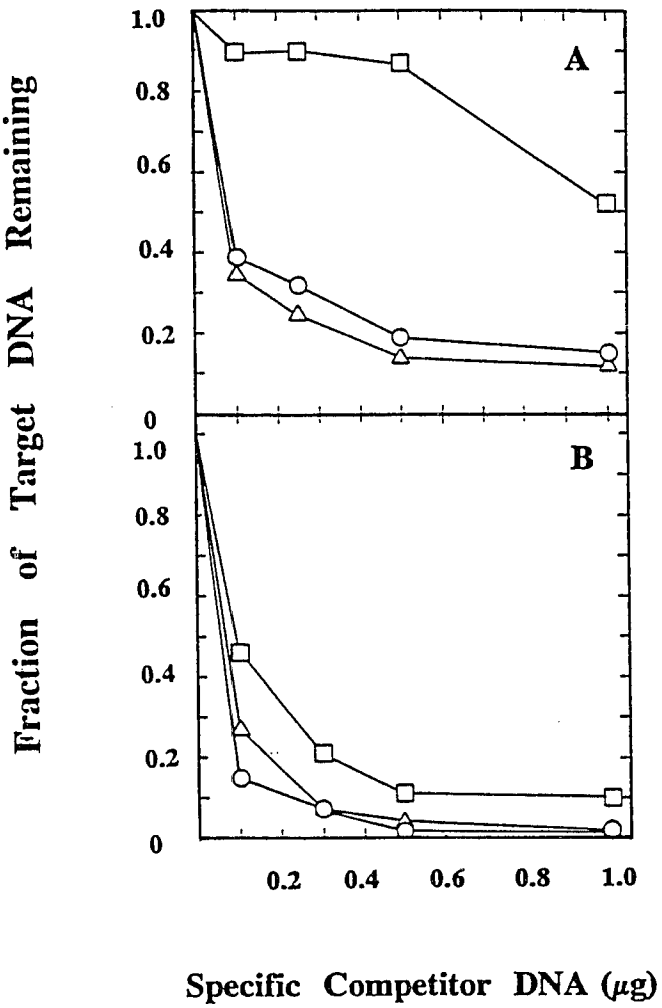


Fig. 8. *E. coli* RNA polymerase binding to λp_L derived promoters as a function of DNA superhelicity. Gel retardation analysis was used to measure the relative affinity of *E. coli* RNA polymerase for the λp_L promoter in the presence of various amounts of unlabeled competitor DNA with the λp_L, Alup_L, or Balp_L promoter. Specific competitor DNAs were added in either relaxed (panel A) or supercoiled (panel B) form. All reactions contained a constant amount of ^{32}P end-labeled DNA fragment containing the λp_L promoter. Specific competitor DNAs with λp_L (□), Alup_L (Δ) and Balp_L (○) promoters were added in the concentrations indicated.

derived from λp_L, the Alup_L and Balp_L promoters, were more effective competitors than the original λp_L. This was most evident when the competitor DNA was relaxed. Since the labeled DNA fragment used in the gel shift assay contained the λp_L, all competition was relative to the original promoter. The addition of curved DNA upstream of the -35 region in the hybrid promoters significantly enhanced binding of *E. coli* RNA polymerase. It should be noted that these results differ from those obtained with the nitrocellulose filter binding assay which failed to demonstrate a difference in the binding of *E. coli* RNA polymerase to these promoters.

The results of the gel retardation assay using a labeled DNA fragment with the original λp_R promoter as the target DNA are presented in Figure 9. For all competitor DNA, the supercoiled forms (Figure 9B) were much more effective in binding *E. coli* RNA polymerase than were the relaxed forms (Figure 9A). In the relaxed form, the Alup_R promoter failed to significantly compete with the original λp_R promoter target DNA. In the same experiment, the Balp_R hybrid and original λp_R promoters were equally effective. Interestingly, in the nitrocellulose filter binding assay, the *E. coli* RNA polymerase bound most tightly to the Alup_R promoter. This may reflect some of the basic differences between the gel retardation assay and the collection of protein-DNA complexes on a nitrocellulose filter. When in the supercoiled form, the hybrid promoters with curved DNA were more efficient competitors than the λp_R promoter. The Balp_R promoter was the most productive as a competitor. This suggests that the binding of *E. coli* RNA polymerase is heightened by the curved DNA when present on a supercoiled plasmid molecule.

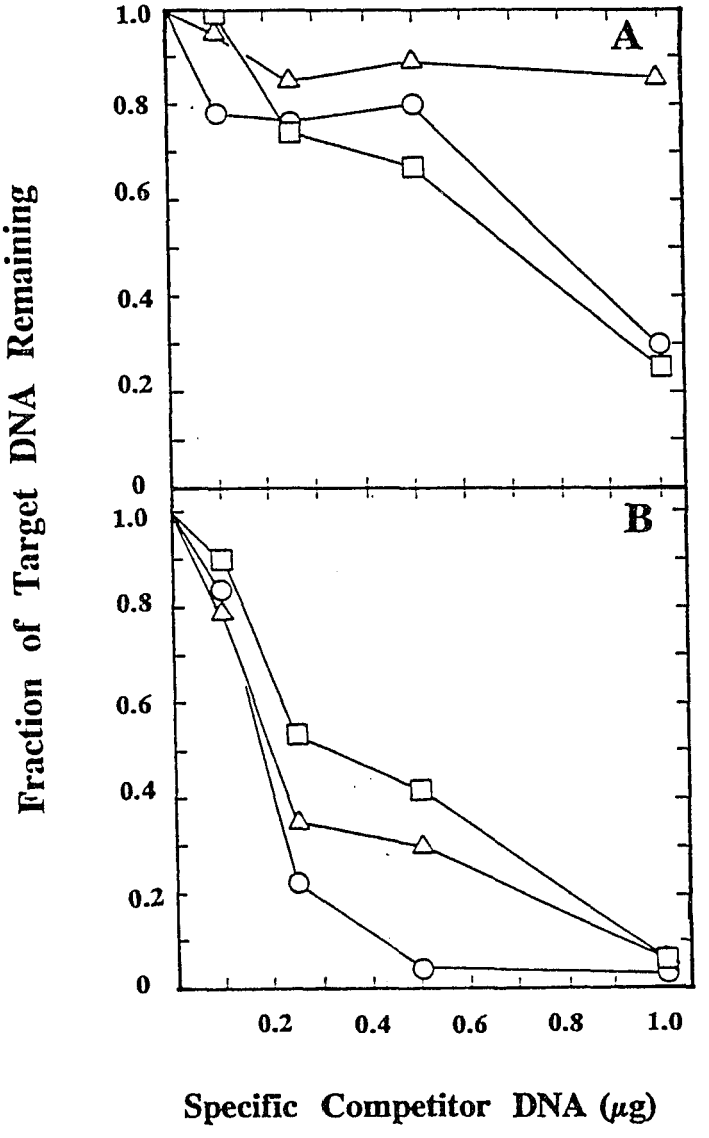
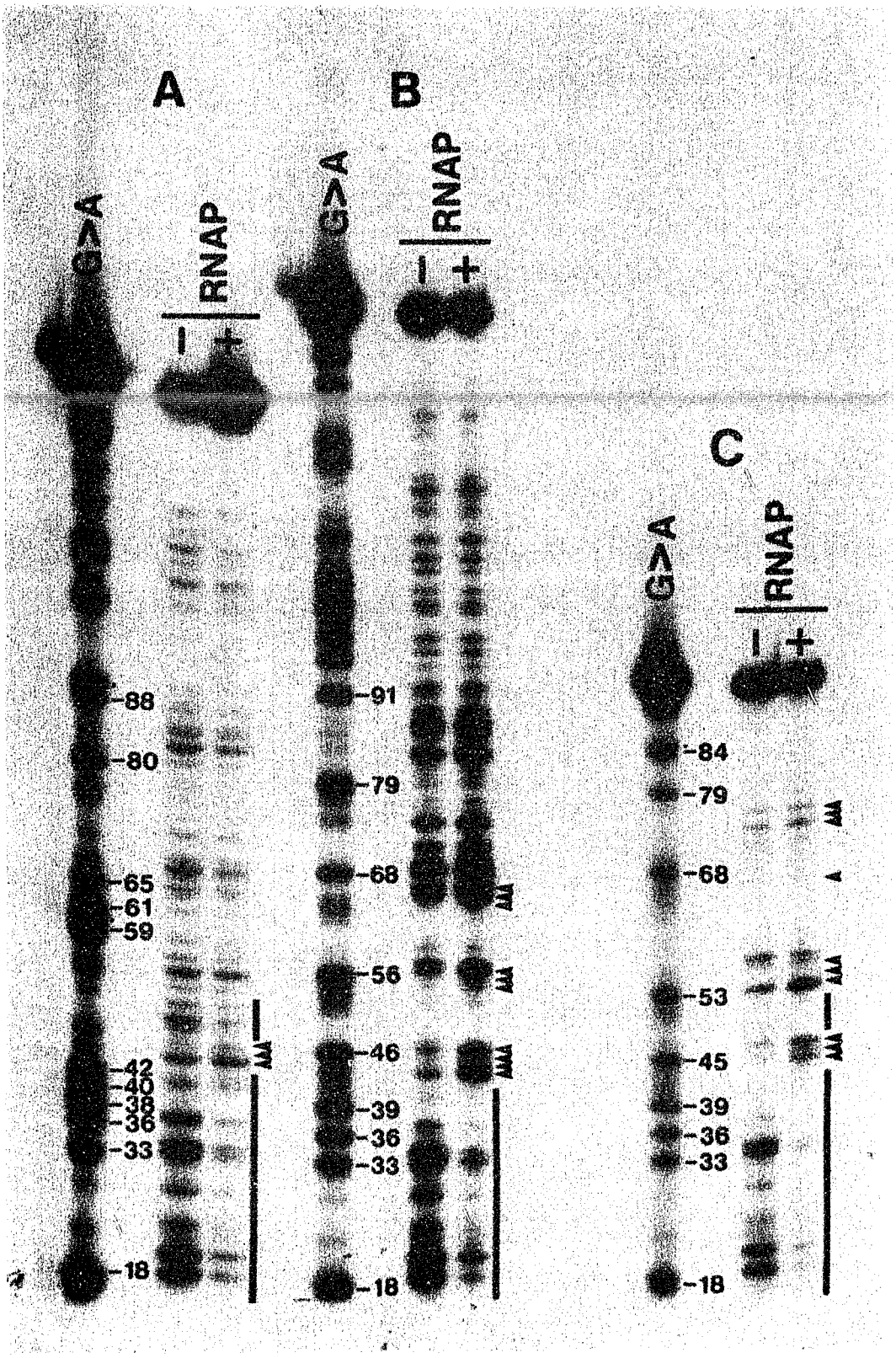


Fig. 9. Effect of DNA superhelicity on *E. coli* RNA polymerase binding to p_R derived promoters. Gel retardation analysis was used to indirectly estimate the relative affinity of *E. coli* RNA polymerase for the λp_R series of promoters. In this assay, end-labeled DNA fragments containing the λp_R promoter were incubated with *E. coli* RNA polymerase in the presence of various amounts of unlabeled specific competitor DNA with the λp_R (□), Alup_R (Δ), or Balp_R (○) promoter. Specific competitor DNAs used in this experiment were either in a relaxed (panel A), or supercoiled (panel B) form. Specific competitor DNAs were added in the concentrations indicated.

DNaseI footprinting analysis of the interactions of *E. coli* RNA polymerase with the λp_R-derived promoters. Analysis of *E. coli* RNA polymerase binding to the λ-derived promoters provided evidence that the curved DNA upstream of the -35 region contributed to enzyme-promoter interactions. This effect might be due to direct interaction between the upstream curved region and the polymerase. Thus, DNaseI footprinting was used as a probe for interactions between upstream regions of DNA curvature and *E. coli* RNA polymerase. The basis of this assay is that cleavage of the phosphodiester backbone of DNA by DNaseI is altered when bound by protein. This may be observed as a footprint or gap in the ladder of DNA fragments if the protein protects the DNA from hydrolysis. Distortion of the DNA helix by DNA bending proteins may lead to regions of DNaseI hypersensitivity as the phosphodiester bonds are made more accessible to DNaseI. The ladder of DNaseI-generated bands is established by electrophoretic separation on denaturing sequencing polyacrylamide gels followed by autoradiography.

Figure 10 corresponds to a representative autoradiogram of the DNaseI footprint obtained for *E. coli* RNA polymerase bound to DNA fragments containing either λp_R, Alup_R or Balp_R. The G > A sequencing reaction was used for each of the promoter-containing DNA fragments to align DNaseI patterns with the nucleotide sequence. For each promoter analyzed, a control lane was provided where the DNA fragments were treated with DNaseI in the absence of *E. coli* RNA polymerase. Lanes marked by a plus sign contained samples bound by *E. coli* RNA polymerase prior to DNaseI cleavage. Guanines in the G > A reaction for each promoter-containing DNA fragment

Fig. 10. DNaseI footprint analysis of *E. coli* RNA polymerase on λp_R-derived promoter-containing DNA fragments. DNaseI footprinting was performed as described in Experimental Procedures. Panel A corresponds to reactions containing λp_R, panel B contains Alup_R, and panel C corresponds to Balp_R. Singly end-labeled DNA fragments containing the promoters λp_R, Alup_R and Balp_R were incubated in the presence (lanes with a plus sign) or absence of (lanes with a minus sign) *E. coli* RNA polymerase. Guanines in the G > A sequencing reaction for the each of the λp_R-derived promoters have been numbered for sequence reference with DnaseI cleavage patterns. Positions corresponding to DNaseI hypersensitive sites are indicated by arrows. Vertical lines indicate regions of protection from DNaseI cleavage.


A
G>A
RNAP
-
+
-88
-80
-65
-61
-59
-42
-40
-38
-36
-33
-18
B
G>A
RNAP
-
+
-91
-79
-68
-56
-46
-39
-36
-33
-18
C
G>A
RNAP
-
+
-84
-79
-68
-53
-45
-39
-36
-33
-18

have been numbered for nucleotide sequence reference. DNA regions strongly protected from DNaseI cleavage were marked by vertical lines. This protection from DNaseI digestion was provided by the binding of *E. coli* RNA polymerase at or near these regions. A strong protection was observed in the -35 region and the flanking regions of each of the promoters, indicative of *E. coli* RNA polymerase-promoter interactions.

When electrophoresed for shorter periods of time, a DNaseI footprint extending to the +15 region was observed. The extent of this protected region was typical for other *E. coli* promoters. Sites of hypersensitivity to DNaseI were marked by arrows. These hypersensitive sites were indicative of structural alterations of the DNA when complexed with *E. coli* RNA polymerase. There was a periodicity of approximately 10 base pairs in the pattern of enhanced cleavage observed with the Alup_R and Balp_R hybrid promoters. This pattern provided evidence that the upstream curvature of Alup_R and Balp_R was bent when wrapped around the RNA polymerase. The location of the sites hypersensitive to DNaseI was at or adjacent to the 5' end of a run of adenines. By all models, this corresponds to the outside of the curve in intrinsically curved DNA. Thus, the DNA was bent by the RNA polymerase in the same direction as the curvature. The footprint analysis of the λp_R promoter with *E. coli* RNA polymerase did not exhibit the periodic pattern of enhanced cleavage observed with the hybrid p_R promoters.

The DNaseI cleavage pattern for the curved DNA without bound protein is distinctive. DNaseI has low activity on templates with runs of purines. In our

experiment, there is strong suppression of DNaseI cleavage at the 3' end of A-tracts, which would be on the interior of the DNA curve. This phenomenon limited our ability to directly probe the A-tracts.

Figures 11-13 represent the densitometric analysis of the DNaseI footprint obtained from the λp_R, Alup_R and Balp_R promoters, respectively, with *E. coli* RNA polymerase. In each of these figures, the densitometric scans of the DNaseI pattern without and with RNA polymerase are presented separately and superimposed. For the superimposed scans, the area under the pattern obtained with RNA polymerase has been shaded. Areas where the shaded profile showed less absorbance (*i.e.* lower peaks) relative to the control indicate that the RNA polymerase has protected the DNA from DNaseI cleavage. Peaks in the shaded profile that were more prominent than those of the control correspond to enhanced cleavage by DNaseI or DNaseI hypersensitive sites. The superimposed densitometric scans in panel C show strong protection from DNaseI digestion in the promoter region extending up to position -43. In each of the three promoters, there is a region of enhanced DNaseI cleavage centered at -46 immediately followed by an additional short region of DNaseI protection around -50. Upstream of this point, the patterns obtained for the hybrid promoters with curved DNA deviate from λp_R. Multiple regions of protein-induced helix distortion, characterized by DNaseI hypersensitive sites, are clearly visible in the upstream regions of the Alup_R (Figure 12) and Balp_R (Figure 13) promoters, but not in the λp_R promoter (Figure 11). This suggests that the upstream curvature is positioning the DNA in such a way as to enhance its wrapping around the polymerase. The 10 base pair periodic phasing of the

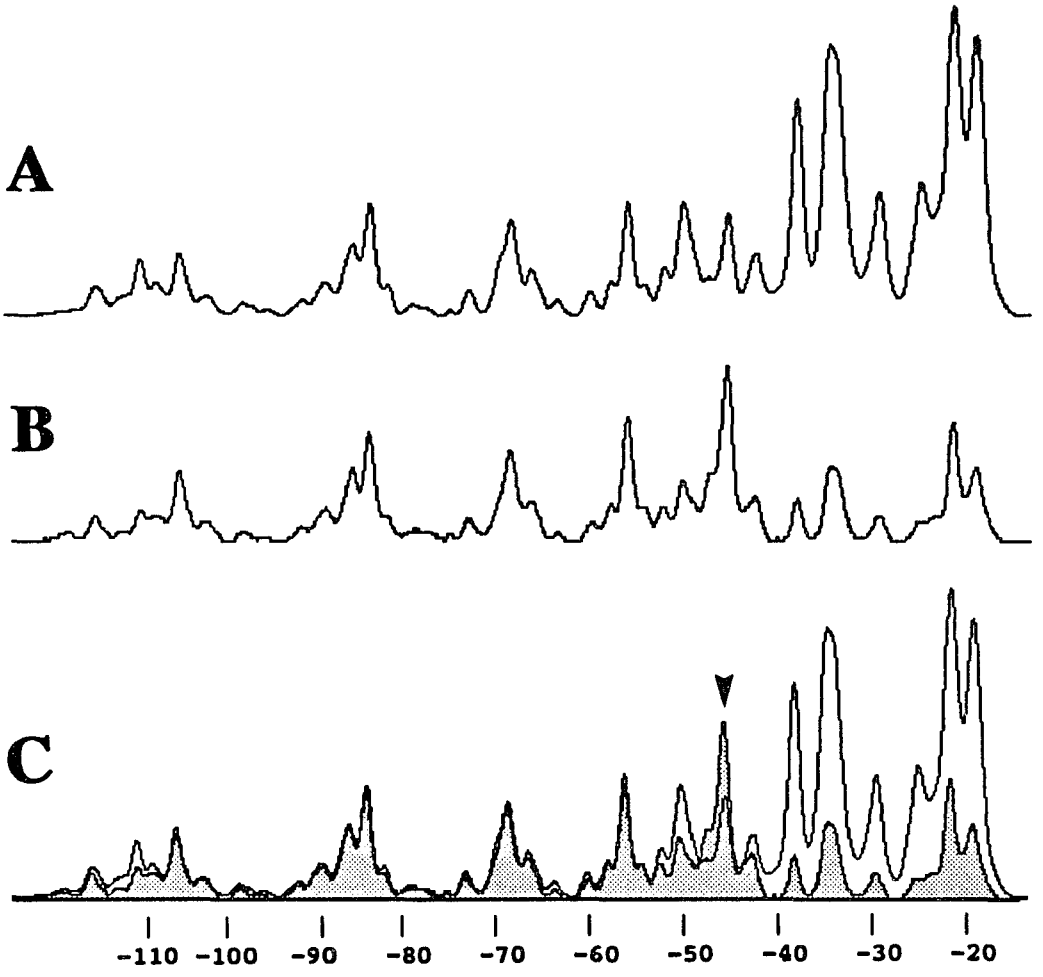


Fig. 11. Densitometric analysis of the DNaseI footprint in the presence and absence of *E. coli* RNA polymerase bound to the λp_R promoter-containing fragment. Panels A and B correspond to the densitometric analysis of the DNaseI footprint obtained for the λp_R promoter in the absence (panel A) or presence (panel B) of *E. coli* RNA polymerase. In panel C, the two densitometric scans have been superimposed to highlight regions of protection and enhanced cleavage. Shaded regions in panel C represent the footprint obtained when *E. coli* RNA polymerase is bound at the promoter. A decrease in the shaded region relative to the control corresponds to protection from DNaseI cleavage. An increase in the shaded region as compared with the control indicates enhanced cleavage by DNaseI. The positions of DNaseI-generated bands are listed in 10 base pair intervals relative to the transcription start site. Arrows correspond to regions of enhanced cleavage by DNaseI.

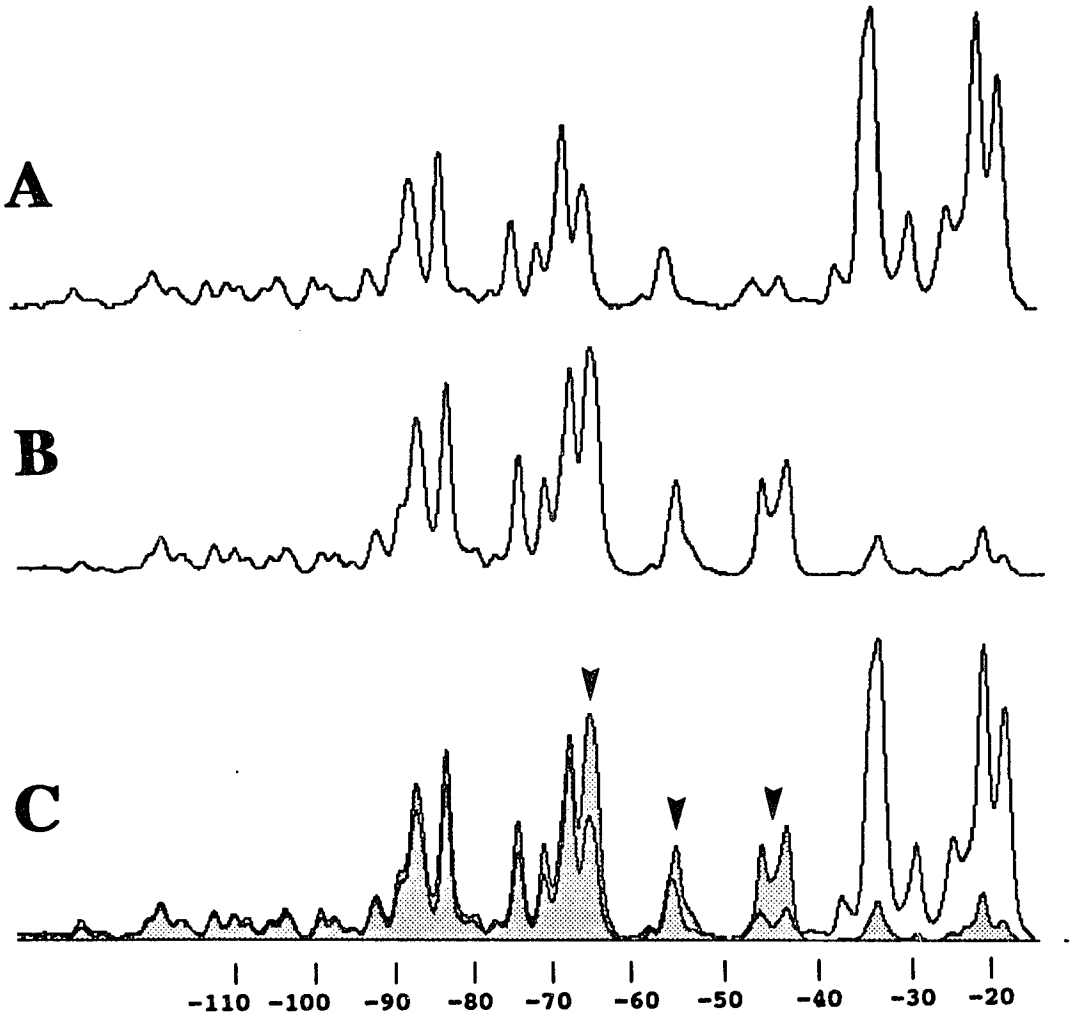


Fig. 12. Densitometric tracings of the DNaseI footprint of *E. coli* RNA polymerase with the Alup_R promoter-containing fragment. Panels A and B correspond to the densitometric analysis of the DNaseI footprint obtained for the Alup_R promoter in the absence (panel A) or presence (panel B) of *E. coli* RNA polymerase. In panel C, the two densitometric scans have been superimposed to highlight regions of protection and enhanced protection and enhanced cleavage. Shaded regions in panel C represent the footprint obtained when *E. coli* RNA polymerase is bound at the promoter. A decrease in the shaded region relative to the control corresponds to protection from DNaseI cleavage. An increase in the shaded region as compared with the control indicates enhanced cleavage by DNaseI. The positions of DNaseI-generated bands are listed in 10 base pair intervals relative to the transcription start site. Arrows correspond to regions of enhanced cleavage by DNaseI.

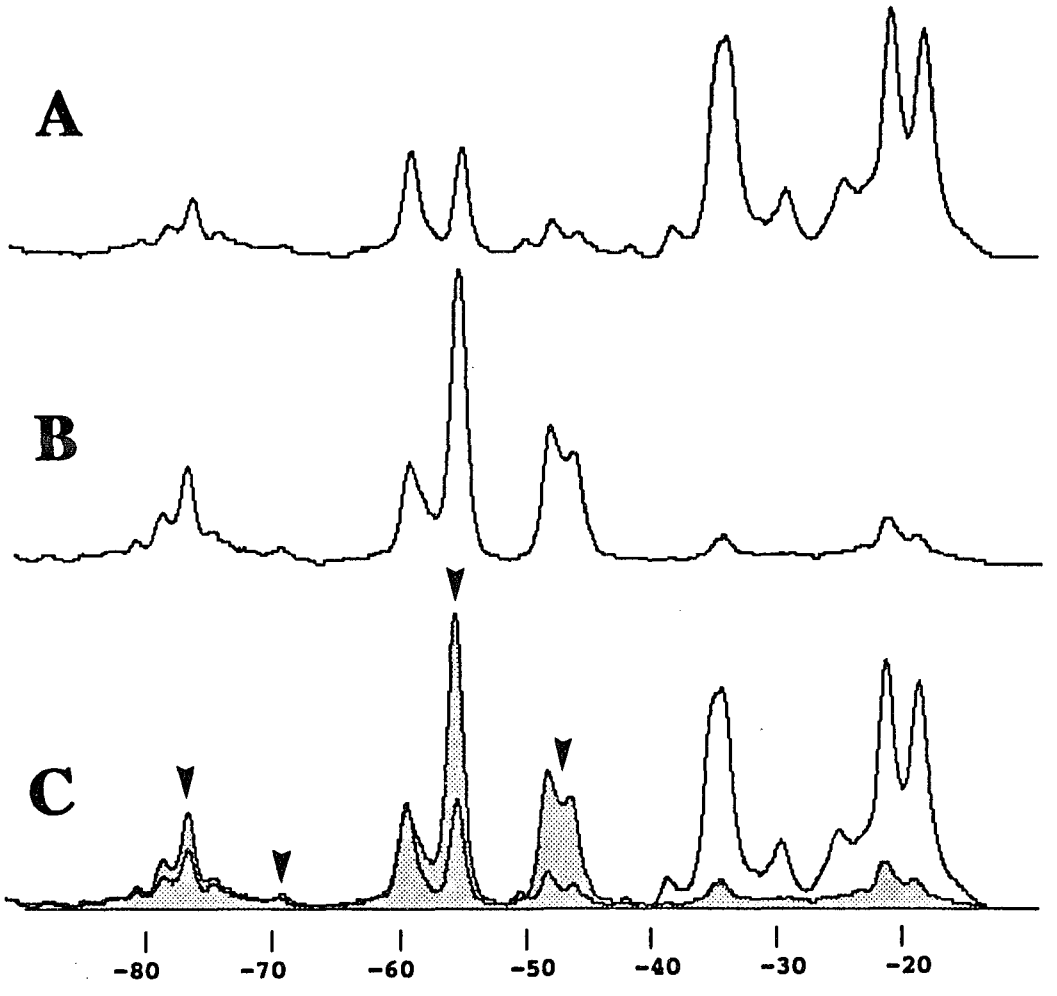


Fig. 13. Densitometric analysis of the DNaseI footprint of *E. coli* RNA polymerase bound to the Balp_R promoter-containing fragment. Panels A and B correspond to the densitometric analysis of the DNaseI footprint obtained for the Balp_R promoter in the absence (panel A) or presence (panel B) of *E. coli* RNA polymerase. In panel C, the two densitometric scans have been superimposed to highlight regions of protection and enhanced cleavage. Shaded regions in panel C represent the footprint obtained when *E. coli* RNA polymerase is bound at the promoter. A decrease in the shaded region relative to the control corresponds to protection from DNaseI cleavage. An increase in the shaded region as compared with the control indicates enhanced cleavage by DNaseI. The positions of DNaseI-generated bands are listed in 10 base pair intervals relative to the transcription start site. Arrows correspond to regions of enhanced cleavage by DNaseI.

pattern of enhanced cleavage was clearly evident for the hybrid p_R promoters (Figures 12 and 13). This pattern was not observed with the λp_R promoter (Figure 11).In addition, results from the densitometric scans of DNaseI footprinting of the hybrid p_R promoters with *E. coli* RNA polymerase indicated that the regions of enhanced DNaseI cleavage extend upstream beyond the region of curvature in the Balp_R promoter (Figure 13). The DNaseI profile for the Alup_R promoter also exhibited regions of enhanced DNaseI cleavage upstream of those centered at -46, -56 and -66. For this promoter, enhanced DNaseI cleavage was observed at several points between -72 and -90. The strict 10 base pair periodicity observed within the curved DNA continued through -75 before deviating. Weak but reproducible enhancements are observed at -72, -75, -80, -87, -88 and -90. The effects at -80 and -90 are out of phase with the 10 base pair periodicity established with the region of curvature.

DISCUSSION

A comparison of the major vegetative RNA polymerases from *E. coli* and *B. subtilis* shows a striking similarity in subunit composition. In addition, both polymerases recognize the same -10 and -35 consensus sequences. Despite their similarities, however, efficient recognition of promoters by *B. subtilis* RNA polymerase appears to require additional structural features other than those required by *E. coli* RNA polymerase. Indeed, *E. coli* RNA polymerase is able to effectively transcribe genes from a wide variety of organisms, including Gram-positive bacteria and yeast (Cohen *et al.*, 1972; Ehrlich, 1978; Struhl *et. al.*, 1976), while RNA polymerase from *B. subtilis* is limited in its ability to express genes from other genera (Ehrlich, 1978). Results from *in vitro* and *in vivo* studies have shown that recognition of *E. coli* promoters by *B. subtilis* RNA polymerase is very inefficient (Lee *et. al.*, 1980; Achberger and Whiteley, 1981; Wiggs, *et. al.*, 1979). One of the distinguishing features of highly utilized promoters in *B. subtilis* is curved DNA immediately upstream of the -35 region of the promoter. This curve is characterized by multiple A tracts in phase with the helical repeat of the DNA.

Effect of curved DNA on promoter function in *B. subtilis*. The association of curved DNA with promoters has been documented in prokaryotes and eukaryotes alike. Some of the most profound effects of curved DNA have been documented in promoters utilized by the *B. subtilis* RNA polymerase. In one study from our

laboratory, curved DNA immediately upstream of the Alu156 and Bal129 promoters had a dramatic effect on the binding of *B. subtilis* σ^A-RNA polymerase and the stability of the complexes formed (McAllister and Achberger, 1988). In addition, the rotational orientation was the critical parameter in the stimulation of *B. subtilis* RNA polymerase binding. Using the Alu156 promoter, the curved DNA could be moved 11 and 21 base pairs from the promoter without a significant loss of promoter function (McAllister and Achberger, 1989). A reduction in promoter function was observed when the curved DNA was displaced from the promoter in increments that deviated from the helical repeat of B-form DNA. Insertions of as few as 3 base pair between the curved DNA and the -35 region dramatically decreased the formation of open promoter complexes (Stemke, 1993).

One of the basic questions asked in the present study was whether the curved DNA found on the Alu156 and Bal129 promoters could affect the transcription of heterologous promoters by the *B. subtilis* RNA polymerase. To explore this question, the curved DNA from the Alu156 and Bal129 promoters was substituted for the DNA sequence upstream of the -35 region in the *E. coli* phage promoters λp_R and λp_L. The spacing present in the Alu156 and Bal129 was preserved with the hybrid promoters, Alup_R, Balp_R, Alup_L and Balp_L.

To evaluate promoter utilization in *B. subtilis*, each of the original λ phage promoters and hybrid promoters were transcriptionally fused to the *cat* gene of plasmid pDMP16. CAT specific activities from these strains indicated that the curved DNA had an affect on utilization of the hybrid promoters. The curved DNA did not increase

promoter utilization in every case. In the Balp_R promoter, a slight decrease in CAT specific activity was observed relative to the original λp_R. The evidence indicated that the curved DNA affected transcription *in vivo* presumably by altering the ability of the RNA polymerase to structure the promoter. The decrease in transcription could be explained (1) if the curved DNA made productive structuring of the promoter less likely or (2) if the curved DNA facilitated RNA polymerase binding and decreased promoter clearance. Promoter clearance is the release of the σ subunit and promoter by the core RNA polymerase during the transition to elongation phase. Changes in the promoter that increase the affinity of the RNA polymerase for it can actually decrease promoter clearance and overall transcription (McAllister and Achberger, 1988).

To test these two possibilities, binding of *B. subtilis* RNA polymerase to these promoters was measured. Since RNA polymerase binding represents the early steps in transcription initiation and the CAT specific activity assays reflect the production of functional mRNA, one would expect agreement between these assays if RNA polymerase binding is the rate limiting step in transcription for that promoter. The shuttle vector pDMP16 was used to make transcriptional fusions since it employs as a reporter gene the *cat* gene from a Gram-positive bacterium that is effectively translated in both *E. coli* and *B. subtilis*. An example where the addition of curved DNA dramatically increased RNA polymerase binding and *cat* gene expression in *B. subtilis* was the Balp_L promoter. The CAT specific activity increased 8 fold over that observed with λp_L.

In the λp_R series, a significant increase in *B. subtilis* RNA polymerase binding was observed in the hybrid promoters containing the curved DNA. However, in transcriptional fusions to the *cat* gene, CAT specific activity decreased with the hybrid promoters. This result is indicative of a sacrifice of promoter clearance for tighter RNA polymerase binding to the promoter. Once the RNA polymerase is bound to the promoter, it cannot effectively release this DNA and enter elongation. This phenomenon has been observed in promoters for early RNA synthesis in *B. subtilis* bacteriophage and in synthetic promoters made using the consensus sequences at the -10 and -35 regions. It appears that in biological systems, maximum promoter efficiency requires moderation in many of the RNA polymerase-DNA interactions.

There were not any promoter constructs where *B. subtilis* RNA polymerase binding was found to significantly decrease while the CAT specific activity increased. This finding would have been consistent with the curved DNA being misaligned with the promoter and actually inhibiting the structuring of the DNA for transcription initiation. For *B. subtilis* RNA polymerase, it appears that the curved DNA influenced the interactions between the enzyme and the promoter.

Curved DNA and promoter function in *E. coli*. Since the addition of curved DNA to the λp_R and λp_L promoters affected their function in *B. subtilis*, would similar effects be observed in *E. coli*? On one hand, one would not expect there to be an effect since phage λ promoters evolved in association with *E. coli*. It would be difficult to improve on the natural system. On the other hand, if curved DNA is an element to

promote high level, constitutive transcription from promoters, as with tRNA and rRNA gene promoters, then curved DNA may affect the function of these promoters.

In the initial analysis of these promoters, the *cat* gene transcriptional fusions were transformed into *E. coli* JM83. The addition of curved DNA to the λp_R and λp_L promoters did not result in a change of CAT specific activity in excess of two fold. There was a small increase in expression when the curved DNA from the Bal129 promoter was substituted for the upstream DNA in the two λ promoters. At this point in the study, the possibility existed that the *E. coli* RNA polymerase was not strongly influenced by curved DNA immediately upstream of the -35 region of the promoter.

E. coli RNA polymerase binding to the original and hybrid promoters was examined using the nitrocellulose filter binding assay to see if there was evidence for altered promoter-enzyme interactions. Addition of DNA curvature had a modest but reproducible effect on *E. coli* RNA polymerase binding in the λp_R series of promoters. The failure to observe a significant difference in RNA polymerase binding with the λp_L series of promoters may relate to the presence of intrinsic DNA curvature in the upstream region of the original λp_L promoter (Giladi *et al.*, 1990). It was reported that deletion of the upstream region of this promoter results in a 2-fold reduction in gene expression. In constructing the hybrid promoters with curved DNA immediately upstream of the λp_L promoter, we may have substituted one transcription enhancing element for another. In addition, we have noted a run a thymines (*i.e.*, an A tract on the complementary strand of DNA) at -25 of the λp_R promoter. This would be predicted to curve the DNA in the same direction as the curved DNA added to the

hybrid promoters. The presence of this second region of curvature may be responsible, in part, for the ability of curved DNA to stimulate *E. coli* RNA polymerase binding to the λp_R hybrid promoters on linear DNA fragments. The failure of the *E. coli* RNA polymerase to discriminate among the various promoter constructs to the same extent as the *B. subtilis* enzyme may indicate (1) that *E. coli* RNA polymerase is not responsive to curved DNA immediately upstream of the promoter, (2) that there is something in *E. coli* that masked the effect of curved DNA on transcription, or (3) that the *in vitro* techniques that we used to analyze promoter function were not versatile enough to analyze promoters on supercoiled DNA templates.

H-NS does not repress transcription from hybrid promoters. A likely candidate for a protein that could mask the effect of DNA curvature is the *E. coli* H-NS protein. Several researchers have demonstrated that the H-NS protein preferentially recognizes curved DNA sequences (Yamada *et al.*, 1990, Yamada *et al.*, 1991, Ueguchi, *et al.*, 1993). H-NS has been characterized as a sequence-dependent DNA-binding protein which is often found associated with regions of curvature. Recently a group of genes were identified that are over-expressed in an *hns* mutant (Yoshida, *et al.*, 1993). H-NS is believed to bind to regions of curved DNA associated with promoters and repress transcription. In the present study, when hybrid promoters with curved DNA were tested *in vivo*, there was no correlation between the presence of curved DNA and stimulation of transcription in an *hns* mutant. Expression from the λp_R and Balp_R promoters was stimulated in the *hns* genetic background, but the λp_R

promoter does not have A-tract DNA curvature. When tested for intrinsic DNA curvature using the polyacrylamide mobility analysis, the λp_R promoter DNA displayed no more curvature than the average bacterial promoter. All promoters in this study contained multiple putative H-NS binding sites. It should be noted, however, that the putative recognition sequence of the H-NS protein is fairly non-specific, with numerous nucleotide substitutions possible. It is presently not known how the site-specific nature of H-NS binding relates to its affinity for curved DNA. It does not appear that H-NS protein is masking the function of the hybrid λ promoters in this study.

Role of DNA supercoiling in the binding of *E. coli* RNA polymerase to hybrid promoters. In a study of CAP activation of the *lac* operon promoter, curved DNA was substituted for the CAP site (Gartenberg and Crothers, 1991). The curved DNA lead to significant transcription stimulation from the *lac*P_1 promoter when tested on a supercoiled template. On a linear, relaxed DNA template, the curved DNA would not substitute for the CAP site and CAP-cAMP activation. The authors proposed a model where the curved DNA defined the end of a superhelical domain and facilitated the wrapping of DNA around the RNA polymerase bound to the promoter. This looping would be strongly inhibited on a linear DNA molecule. The active CAP-cAMP complex is believed to bend the DNA around the RNA polymerase in a fashion consistent with the proposed model. If DNA supercoiling is necessary for curved DNA to enhance transcription from promoters, then the filter binding assay which used linear DNA fragments would have missed the effect.

To test for an effect of DNA supercoiling on *E. coli* RNA polymerase binding, we devised a competition assay using gel retardation analysis. RNA polymerase binding to end-labeled, linear DNA fragments of the original λp_R and λp_L promoters was competed using relaxed (*i.e.*, plasmid DNA linearized by restriction enzyme digestion) or supercoiled molecules containing the same promoter or one of the hybrid promoters with curved DNA. The advantage of this assay over the filter binding assay is that it detects protein-DNA complexes too unstable to be retained on a nitrocellulose filter. In addition, by testing binding to the various promoters using a competition assay, supercoiled templates could be assayed in direct comparison to linearized templates. At our lowest concentration of competitor DNA, there are approximately twice as many unlabeled competitor promoters as the labeled promoter.

It was not surprising that promoters on supercoiled DNA templates were more effective in binding RNA polymerase (*i.e.*, were the most effective specific competitors) in the assay. In general, many promoters are stimulated by the negative supercoiling of the bacterial DNA. The hybrid promoters made with the λp_L promoter effectively competed with the λp_L promoter for RNA polymerase whether on a linear or supercoiled DNA template. As may be expected, the λp_L promoter was an effective competitor against itself only when in the supercoiled form. This experiment provided strong evidence that the curved DNA stimulated binding of the RNA polymerase to the λp_L promoter.

In the λp_R series, the hybrid promoters were effective when on a supercoiled template, but were not as effective on linear DNA fragments relative to the original

λpR promoter. On a linear template, the Alup_R hybrid promoter failed to compete with the λpR promoter. This was surprising since in the nitrocellulose filter binding assay, the *E. coli* RNA polymerase had the greatest affinity for the Alup_R promoter. In both assays, the two promoters, Alup_R and λp_R, were directly competing for RNA polymerase. The difference in these results may reflect the basic difference between the assay techniques. The filter binding assay directly measures those complexes stable enough to be collected on a nitrocellulose filter. The gel retardation assay will detect even weakly bound protein-DNA complexes. It appears that the association rate of *E. coli* RNA polymerase with the λp_R promoter on linear DNA was greater than that with the Alup_R promoter. However, once formed the promoter-RNA polymerase complexes formed with the Alup_R promoter were the most stable. If this were true, there may be a distinct difference in how the RNA polymerase interacts with these promoters.

In every case, the hybrid promoters with curved DNA were bound by RNA polymerase more effectively than the original λ promoters when the DNA templates were supercoiled. This agrees well with the findings of Gartenberg and Crothers (1991). These studies suggest that if the curvature of the DNA is facilitating productive *E. coli* RNA polymerase-promoter interactions, it is much more efficient on a negatively supercoiled DNA template. The curved DNA would define the end of a supercoiled domain and place the RNA polymerase-promoter complex in a loop of the DNA. This would suggest that the curved DNA plays a role in the structuring of the promoter by RNA polymerase.

It appears that the curved DNA added to the λ promoters affected the initial binding of *E. coli* RNA polymerase (*i.e.*, the formation of a closed promoter complex). However, initial RNA polymerase binding may not be the rate limiting step in transcription from the hybrid promoters. Another kinetic step leading to transcription initiation (*e.g.*, strand separation or promoter clearance) may become rate limiting in the presence of the curved DNA. The nitrocellulose filter binding assay, which effectively measures the most stable enzyme-DNA complexes would miss an effect of curved DNA if it affected initial binding, structuring of the DNA, and formation of relatively weak closed promoter complexes. These complexes are effectively detected with the gel retardation assay.

***E. coli* RNA polymerase interaction with DNA upstream of the -35 region.** Results from gel retardation and filter binding analysis suggested that *E. coli* RNA polymerase interacts differently with λp_R and the hybrid promoters with DNA curvature upstream of the -35 region. DNaseI footprinting was chosen to examine the direct effect of *E. coli* RNA polymerase binding on the λ-derived promoters. Since nitrocellulose filter binding analysis indicated that *E. coli* RNA polymerase bound the λp_R hybrid promoters more efficiently than the original λp_R, the λp_R series of promoters were chosen for footprinting with *E. coli* RNA polymerase.

The results from footprinting analysis with the p_R hybrid promoters provided us with evidence for a structuring of the DNA by RNA polymerase. Specifically, this structuring appeared as the wrapping of curved DNA around the RNA polymerase in

a nucleosomal-like fashion with the DNA bent in the same direction as the intrinsic curve. This DNA wrapping was characterized by sites hypersensitive to DNaseI cleavage which were repeated every 10 base pairs in the region of the curved DNA (Figure 14). This periodicity of sites exposed to DNaseI is similar to that observed by Hayes *et al.* (1990), who used DNaseI and hydroxylradical footprinting to characterize the interaction of the *Xenopus borealis 5S RNA* gene with the histone core. The authors demonstrated that the regions of maximum DNaseI and hydroxyl radical cleavage of the DNA in nucleosomal form were repeated with a helical periodicity of approximately 10.18 base pairs per turn. With *E. coli*, this pattern of DNaseI hypersensitivity was observed with the bending of DNA by the CAP-cAMP complex at the *lac*P1 promoter (Spassky, *et al.*, 1984).

Recently, Rees *et al.*, (Rees *et al.*, 1993) used scanning force microscopy to image *E. coli* RNA polymerase complexed with the λp_L promoter. The authors provided evidence that the DNA is bent when *E. coli* RNA polymerase is bound at the promoter during open complex formation. We propose that the addition of upstream curvature to the λp_R promoter facilitated the normal wrapping of DNA around the RNA polymerase and placed high percentage of promoters in this structured conformation so that it was detected by DNaseI footprinting. It is possible the bending of the DNA around *E. coli* RNA polymerase is a mandatory but elusive step in the formation of the initiation complex. A similar model of DNA wrapping has been proposed for transcription activation by the CAP-cAMP complex (Gartenberg and Crothers, 1991).

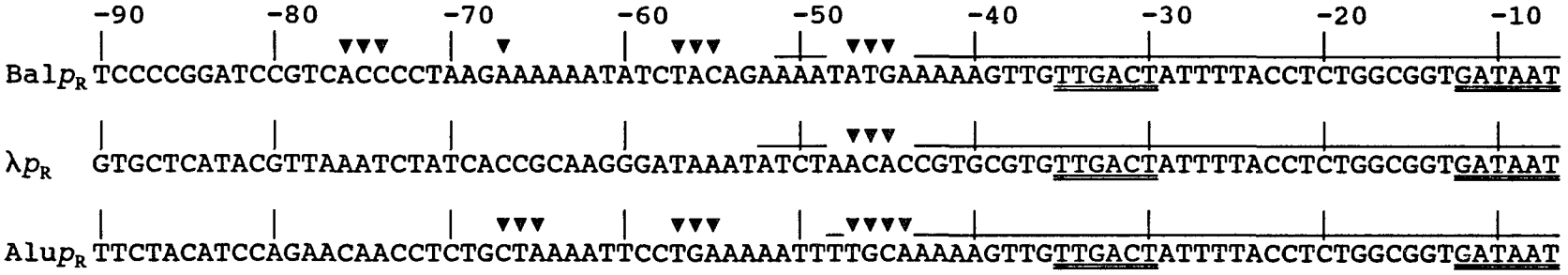


Fig. 14. DNaseI data relative to the nucleotide sequence for the λp_R series of promoters. Lines above the nucleotide sequence represent protected regions, while arrows represent sites hypersensitive to DNaseI cleavage. The -10 and -35 regions have been double underlined.

Models for the role of curved DNA in transcription. For the *B. subtilis* RNA polymerase, our laboratory has entertained the model that the curved DNA upstream of the -35 region of the promoter facilitates RNA polymerase binding. The curvedsequence was proposed to loop the DNA upstream of the promoter back to the RNA polymerase and permit binding of the enzyme. This model was supported by the finding that the A-tract DNA could be moved (*i.e.*, by double strand oligonucleotide insertions) 11 and 21 base pairs away from the promoter without significant loss of promoter function (McAllister and Achberger, 1989). When the curve was moved in increments other that the 10-11 base pair helical repeat of B-form DNA, promoter function was impaired. The rotational orientation and not the linear displacement of the curved DNA was the most critical factor in stimulating transcription. In the present study, the addition of curved DNA to heterologous promoters affected their utilization in *B. subtilis*. In most cases, the addition of curved DNA increased the affinity of *B. subtilis* RNA polymerase for the promoter.

Recently a new model was proposed to explain the enhanced promoter function observed when DNA rich in (A+T) is located upstream of the -35 region. Using the *E. coli rrnB*P_1 promoter, Gourse and associates (Ross, *et al.*, 1993) provided direct evidence for binding of the RNA polymerase α subunit to the DNA rich in (A+T). This binding accompanied a 30-fold increase in transcription from this promoter. The authors go on to suggest that other promoters that have DNA rich in (A+T), including those with A tracts, may also interact with the α subunit. However, the upstream region of the *rrnB*P_1 promoter does not have multiple A tracts with the 10-11 base pair

periodicity indicative of curved DNA. It is currently unknown whether α subunit binding requires a specific nucleotide sequence or simply DNA rich in (A+T).

In our study, we find a short region of protection from DNaseI around -50 on each of the promoters in the λp_R series. This is the same general area characterized by DNaseI footprinting as the A + T rich α subunit binding site on the *rrnB*P_1 promoter (Ross, *et al.*, 1993). Each of the λp_R-derived hybrid promoters has an A tract at this position and the original λp_R promoter has a 12 base pair A + T rich sequence at this site. While we cannot preclude a role of direct binding of the RNA polymerase to this site, in the presence of curved DNA, we have evidence for much more extensive interactions upstream of this site. There was no evidence of DNA structuring *via* wrapping in the work with the *rrnB*P_1 promoter or in our work with the λp_R promoter. The extensive DNA wrapping observed for promoters with curved DNA is sufficient to negate the suggestion by Ross, *et al.*, (1993) that enhanced RNA polymerase binding ascribed to curved DNA was attributable to the same α subunit binding effect observed for the *rrnB*P_1 promoter. Our DNaseI footprinting data is the first direct evidence that the RNA polymerase wraps the DNA around itself.

To wrap the DNA around the RNA polymerase, the enzyme must bind the DNA at sites outside the -10 and -35 region. From the present study, it is unclear whether the RNA polymerase directly binds the curved DNA or DNA upstream of the curved DNA. The presence of alterations in the DNaseI digestion pattern upstream of the curved DNA in the hybrid λp_R promoters indicates that the RNA polymerase binds this DNA to some extent. If this is true, then the curved DNA would serve, in part, to

loop the DNA back to the RNA polymerase as proposed previously (McAllister and Achberger, 1989). We predict that DNA curvature upstream of the promoter sequence structures the DNA and thus provide additional contact(s) between *E. coli* RNA polymerase and the DNA template.

Curved DNA failed to affect promoter utilization in *E. coli* cells to the same extent as in *B. subtilis* cells. It is possible that the step or steps in transcription initiation stimulated by curved DNA were not the rate limiting step for the λp_R and λp_L promoters in *E. coli*. Secondly, the DNaseI footprinting experiments demonstrate that the curved DNA stabilized the RNA polymerase in a complex with the DNA wrapped around it. It is distinctly possible that by stabilizing the wrapped complex, the curved DNA may inhibit the transition to the next step in transcription. For example, by increasing the affinity of the RNA polymerase for the promoter, it is possible to reduce promoter clearance (*i.e.*, release of the promoter in the transition to the elongation complex).

Current models for the interaction of *E. coli* RNA polymerase with the promoter leading to transcription initiation speculate, without evidence, that the DNA is wrapped about the enzyme at step prior to open promoter complex formation. The present study provided solid evidence for the wrapping of DNA upstream of the promoter around the RNA polymerase. Furthermore, the addition of curved DNA which stabilized the wrapped complex, stimulated the initial binding of *E. coli* RNA polymerase to the λp_R and λp_L promoters. This study provides the first direct evidence in the support of the

models citing the contribution of DNA structuring by RNA polymerase to the initiation of transcription.

CONCLUSIONS

Throughout the course of this research several important findings were made. The addition of curved upstream DNA sequences from Bal129 and Alu156 was able to significantly enhance the binding affinity for *E. coli* RNA polymerase and *B. subtilis* RNA polymerase to promoters on linear fragments. Specifically, the hybrid promoters Alup_L and Balp_L demonstrated an enhanced binding affinity for *E. coli* RNA polymerase relative to the original λp_L promoter. To our knowledge, this is the first demonstration of DNA curvature located immediately upstream of the -35 promoter region affecting *E. coli* RNA polymerase binding to a linearized template.

For the promoters tested in this study, efficient promoter utilization in *B. subtilis* was more dependent on the presence of upstream DNA curvature than in *E. coli*. Replacing the upstream region of the λp_R and λp_L promoters with that of Alu156 and Bal129 dramatically altered promoter utilization in *B. subtilis*. The addition of the Alu156 and Bal129 upstream sequences to the λp_L promoter resulted in four and eight-fold increases in *in vivo* expression, respectively, in *B. subtilis*. However, utilization of the λp_R promoter by *B. subtilis* RNA polymerase was decreased by addition of the curved upstream sequence. This suggests that the promoter sequences themselves (*i.e.*, the -10 and -35 regions) play an important role in the proper structure of the promoter for efficient utilization. Although similar in sequence, the two promoters have single base pair differences in the -10 and -35 regions. Possibly of greater importance, the λp_R promoter has a thymine tract at -25 that is in phase with the A tracts in the curved

DNA of the hybrid promoters. These thymines are not present in the λp_L promoter and may affect the structuring of the promoter. In addition, the upstream curved sequences from Alu156 and Bal129 are not identical, and it is therefore not surprising that they affected *in vivo* expression and *in vitro* binding of RNA polymerase differently when ligated upstream of the λp_R and λp_L promoters.

The *E. coli* H-NS protein is a site specific DNA binding protein which binds preferentially to regions of DNA curvature. We hypothesized that the H-NS protein could bind preferentially to hybrid promoters with curved DNA, thus negating a positive contribution to RNA polymerase binding. The H-NS protein did not significantly influence the ability of DNA curvature to affect transcription from the hybrid promoters tested. Although the H-NS protein did repress transcription from the λp_R and Balp_R promoters, the effect was not dependent on DNA curvature, since the λp_R promoter contains no upstream in-phase A tracts. Further work is necessary to completely elucidate the role of H-NS on curved DNA upstream of the λp_R and λp_L promoters.

Relative to the original λp_R promoter, superhelicity enhanced the ability of λp_R hybrid promoters to act as competitors for binding *E. coli* RNA polymerase in the gel retardation assay. However, the λp_L hybrid promoters containing curvature served as effective competitors on both supercoiled and linear DNA molecules, again demonstrating the ability of curved DNA on linear templates to affect binding by *E. coli* RNA polymerase.

Results from DNaseI footprinting experiments suggested that the addition of curvature immediately upstream of the -35 region of the λp_R promoter structured the DNA by wrapping it around the *E. coli* RNA polymerase in a tight nucleosomal-like fashion. A direct consequence of this structuring may be the formation of additional contacts between the polymerase and the DNA. Although DNA bending proteins, such as CAP, are known to structure the DNA in an analogous manner upon binding, this is the first demonstration of the wrapping of DNA around *E. coli* RNA polymerase without the aid of a DNA bending protein.

In our study, the enhanced binding of *E. coli* RNA polymerase to regions with curved DNA was consistent with the DNA looping model, where upstream DNA is wrapped around the RNA polymerase to provide for additional contacts between the DNA and the enzyme. Additional work must be done to determine if there is a direct interaction between our hybrid promoters and the α subunit of *E. coli* RNA polymerase. The use of mutant derivatives of the α subunit of the *E. coli* RNA polymerase holoenzyme with our hybrid promoters should definitively address this question.

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VITA

Cheryl Anne Nickerson was born on October 19, 1961 in Scottsdale, Arizona. Her family later moved to Milwaukee, Wisconsin, where, in 1979, she graduated with honors from Bay View High School. She attended Tulane University in New Orleans, Louisiana, from 1979-1983, and graduated with a B.S. degree in Biology. She was then accepted into the graduate program of the Genetics Department at the University of Missouri in Columbia, Missouri, where she received her M.S. degree in May of 1988. While at the University of Misssouri, she was awarded the Outstanding Graduate Teaching Assistant in the Department of Biology and in the School of Arts and Sciences. In addition, her Master's thesis, entitled "Structure-Function Studies of tRNA", received public forum at the 1987 International Symposia for tRNA research, held in Umea, Sweden.

In August of 1988, she enrolled in the doctoral program in the Department of Microbiology at Louisiana State University. Portions of her doctoral research have received public forum at the 1992 and 1993 national meetings of the American Society for Microbiology. Cheryl is currently attending Louisiana State University, where she is a candidate for the Doctor of Philosophy degree in Microbiology.

DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Cheryl Anne Nickerson

Major Field: Microbiology

Title of Dissertation: Role of Curved DNA in Promoter Recognition with Bacillus subtilis and Escherichia coli RNA Polymerase

Approved:

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

Date of Examination:

January 20, 1994